Meddling with *Marinobacter*: Microbial Interactions with the Environment

A DISSERTATION
SUMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

Benjamin M. Bonis

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Jeffrey A. Gralnick: Advisor

June 2018

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Acknowledgements

How could I possibly express the profound gratitude towards all those who have helped me reach this point? Moments of inspiration, illuminating conversation, and thoughtful insights that outwardly seemed just the mundane passing of time; but cumulatively provided the knowledge, direction, and perspective that led me here. Thank you to those who guided my education, both in the professional capacity as professors and mentors, and as my lab mates and peers. Special thanks of course to my advisor, Jeffrey Gralnick, for giving me the chance to work in his lab and continue my education, as well as fostering such a wonderful community to work in. Special thanks as well to Daniel Bond for his mentorship and remarkable ability to make every conversation a learning experience. To the lab mates who helped me navigate the difficulties of life and graduate school, and made it a great time along the way. Special thanks to Zara Summers, Nick Kotloski, Abhiney Jain, and the Erics; they have no idea how influential they have been.

I owe much of my achievement to my eternally supportive family, who inspired, taught, and molded me towards my goals. It is the unique combination of all of your contributions and talents that made me who I am. Thank you and I love you.

Dedication

I dedicate this thesis to my wife, Jamie Bonis. You have been my greatest motivation and dearest friend, and for that this accomplishment is as much yours as mine. Thank you for sharing your life with me and for your enduring support.

Abstract

Despite inhabiting a wide variety of biomes and conditions, relatively little work has been devoted to the genus *Marinobacter* beyond isolation and initial characterization. The broad range of tolerances displayed by the *Marinobacter* suggest a metabolically diverse clade with great potential for biotechnological use and environmental study. In addition to hydrocarbon degradation and the synthesis of wax esters, many strains have been found to influence the redox state of metals and oxidize cathodes. Ubiquitously found and readily cultivated, a surfeit of genomic sequences and metadata are available for this genus, though little effort devoted to curation during deposition. Here we utilize 71 public and private *Marinobacter* genomes to curate the taxonomic and phylogenetic status of these strains, and use comparative genomics to assay the core and pangenome content and how it relates to the demonstrated plasticity of this genus. We find a number of strains erroneously assigned or lacking species identifiers, and offer an updated taxonomy. We also find a deeply branching clade of psychrophilic *Marinobacter*, suggesting a dearth in the continuity of sequence space of the genus.

Due to their extensive distribution and abundance, of particular interest regarding the *Marinobacter* is the contribution to elemental cycling through extracellular redox chemistry. Utilizing a novel species of *Marinobacter* isolated from the Soudan Iron Mine in northern Minnesota, we develop a model system to better interrogate the interactions of *Marinobacter* with the environment. Characterization, development of a robust genetic system, and the establishment of *Marinobacter subterrani* strain JG233 as an Fe(II)-oxidizing bacterium additionally provides a model organism for the assessment of

environment-microbe interactions. Using the techniques optimized in *Marinobacter subterrani*, genetic systems were developed for an additional 6 strains of *Marinobacter* to assess essential gene profiles across a genus using transposon mutant libraries. Comparative essential gene profiling using strains from diverse biomes of isolation provides the foundational tools to begin assessment of the effect of environment on gene content, regulation, and conditional essentiality.

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Chapter 1: An Overview of the Genus *Marinobacter* and Fe(II)-Oxidizing Organisms

The Genus Marinobacter

Bacteria of the genus *Marinobacter* are found ubiquitously in aquatic and terrestrial salinous environments. As a genus, the *Marinobacter* harbor the capacity to tolerate a tremendous range of conditions; from hydrothermal vents (Wang et al., 2012) to the Antarctic (Liu et al., 2012a; Mikucki and Priscu, 2007), surface-ocean (Kaeppel et al., 2012) to hypersaline lakes (Bagheri et al., 2013), and industrial waste including frack water effluent (Daly et al, 2016) and a vineyard evaporation pond (Liebgott et al., 2006). Plasticity is characteristic of the broad capabilities of individual species, as well as the genus as a whole. First characterized as hydrocarbon-degrading bacteria (Gauthier et al., 1992), interest in the *Marinobacter* began focusing on use as a bioremediation tool in oil-contaminated areas (Wong et al., 2002). Isolation of additional species and the concomitant discovery of the breadth of the metabolic pathways observed in the genus poises the *Marinobacter*acae as potent and influential organisms in the future of biotech. Production of wax esters and tolerance to a wide variety of harsh conditions make the *Marinobacter* a promising platform for biosynthesis, while native ability to grow prototrophically on a variety of carbon sources and degrade recalcitrant materials lends to use in bioremediation. Robust environmental tolerance and a wide array of growth conditions allows many *Marinobacter* to be easily cultivated and thus act as ideal candidates as model organisms and chassis for biotechnology. Several species have been observed to alter the redox state of extracellular metals (Bonis and Gralnick, 2015; Wang et al., 2012), suggesting several *Marinobacter* may be capable of lithotrophy as well. Recent evidence suggests several species are able to electronically interact with

electrodes, providing a metabolically flexible interface between biology and technology (Rowe et al, 2015; Wang et al., 2015).

Fe(II)-Oxidizing Microbes

Iron-oxidizing microbes are organisms capable of facilitating the removal of an electron from reduced iron species. As the solubility of iron is largely dependent on redox state, and can have a profound effect on iron bearing minerals, elemental cycling, and anthropogenic iron-bearing structures. Classically studied iron-oxidizing bacteria primarily convert soluble Fe(II) to Fe(III), accelerating the rusting of iron-bearing structures as well as resulting in copious quantities of insoluble Fe(III)-precipitates. Both are particularly evident in municipal water supplies, where plumbing is simultaneously deteriorating and becoming occluded by the activity of Fe(II)-oxidizing bacteria, although most occurrences where iron-bearing structures interact with water are impacted (Emerson et al., 2010). However, conversion of Fe(II) is crucial to iron cycling, an elemental cycle directly effecting the cycling of other key elements including carbon, oxygen, and nitrogen; as well as a lesser role in the sequestration and release of other elements (Straub et al., 2004; Rohwerder et al., 2003). Possibly of greatest interest is the potential of Fe(II)-oxidizing bacteria in biotechnology. Already implemented in biomining, additional applications may include electrobiological interfacing as well as biofuel production.

The ferroxidase, or the component directly facilitating the conversion of Fe(II) to Fe(III), and the mechanism of electron transfer across electrically-insulating cell membranes are of primary interests regarding the Fe(II)-oxidizing bacteria. Because

Fe(II) at neutral pH is soluble, and Fe(III) tends to precipitate, cells that facilitate this conversion at a scale to support life must do so extracellularly, as intracellular precipitates would prove lethal and export would be energetically unviable. Even occurring extracellularly remains problematic as cells risk entombment by the metabolic product, Fe(III). Additionally, the rate of abiotic Fe(II) oxidation increases exponentially with molecular oxygen concentration in environments of neutral pH. Rapid abiotic oxidation easily outpaces biotic rates, making microbial Fe(II)-oxidizers unable to complete and survive. There are four main mechanisms microbes employ to mitigate these complications, and are additionally used to group them; acidophilic, nitrate reducing, phototrophic, and microaerobic.

Fe(II) Oxidation Systems

Protons are a product of abiotic Fe(II) oxidation, as described in the rate reaction of $-\Delta[Fe^{2+}]/\Delta t = k[Fe^{2+}][O_2]\,[H^+]^{-2}$, meaning an exponential decrease in rate accompanies a drop in pH, stabilizing Fe(II) even in oxygen-rich environments (Emerson et al., 2010). At pH 3 and lower, Fe(III) remains soluble, providing an additional benefit to the acidophilic Fe(II)-oxidizing bacteria that couple the oxidation of Fe(II) to the reduction of molecular oxygen in low pH environments. However, the concentration of protons is positively correlated with reduction potential of the Fe(II)/Fe(III) couple, decreasing the potential difference between the electron donor and acceptor, and thus the work that each electron can achieve. The increase in donor potential not only increases the ratio of Fe(II) oxidized per work achieved, it also limits potential electron acceptors to those with relatively more positive potentials. Despite the inefficiency of acidophilic Fe(II)

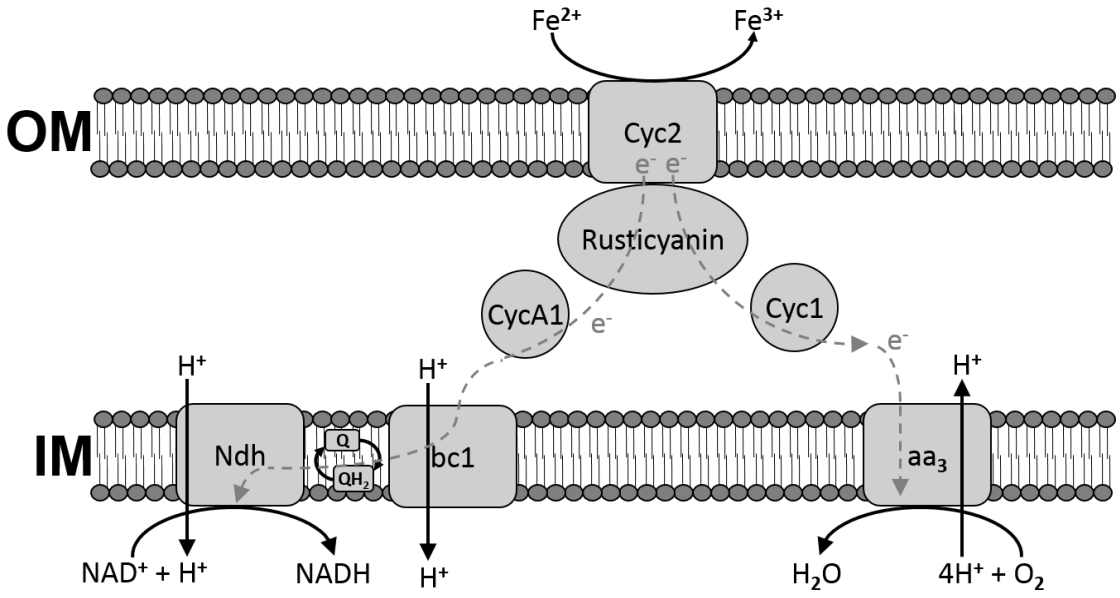

Figure 1.1. Model for Fe(II)-oxidation in *Acidothiobacillus ferrooxydans*. Dotted lines follow proposed electron flow from Fe(II) to inner membrane components. Cytochrome Cyc2 acts as the ferroxidase, removing an electron from Fe(II) and passing it to the cupridoxin rusticyanin. The electron is then passed to one of two periplasmic electron carriers, CycA1 or Cyc1. Depending on the cells energetic requirements, the electron may flow through reverse electron transport to a reducing equivalent, or ultimately reduce oxygen at a terminal oxidase, transporting a proton across the membrane. Abbreviations: OM, Outer Membrane; IM, Inner Membrane; Ndh, NADH Dehyrogenase; aa_3, aa_3-type oxidase; Q, Ubiquinone; QH_2, Ubiquinol.

oxidative metabolism, ease of cultivation and isolation from iron-rich media has progressed the proposed mechanism for acidophilic Fe(II) oxidation in the model organism *Acidothiobacilus ferrooxydans* (Figure 1)(Appia-Ayme et al., 1999). A periplasmic monoheme cytochrome, Cyc2, acts as the putative ferroxidase, transferring electrons from Fe(II) to the periplasmic blue copper protein Rusticyanin (Appia-Ayme et al., 1999). Rusticyanin is then thought to distribute electrons to periplasmic cytochromes CycA1 and Cyc1, directing electron flow either uphill or downhill, depending on the cells energetic needs (Appia-Ayme et al., 1999).

Fe(II)/Fe(III) redox couples suitable to support Fe(II) oxidizing organisms around neutral pH range from roughly –200 mV to +200 mV verses a standard hydrogen electrode. Facilitating Fe(II) oxidation in the absence of molecular oxygen allows nitrate

reducing and phototrophic Fe(II) oxidizing microbes to take advantage of lower potential donors at circumneutral pH while avoiding competition with abiotic Fe(II) oxidation. Similar to the acidophilic Fe(II) oxidizing microbes, nitrate reducing Fe(II) oxidizers sacrifice moving electrons through a greater potential difference to avoid abiotic oxidation complications, though the mechanism of Fe(II) oxidation remains elusive. Though many well-studied bacteria seem to display mixotrophic Fe(II) oxidation coupled to the reduction of nitrate, it is not clear if it is an electrogenic process, or merely an artifact as the products of nitrate reduction can facilitate Fe(II) oxidation. The only organism displaying ferrolithoautotrophic growth reducing nitrate is the currently genetically intractable archaeon *Ferroglobus placidus* (Hafenbradl et al., 1996), calling to question whether bacteria are capable of metabolic nitrate-reducing coupled Fe(II)-oxidation at all (Klueglein and Kappler, 2013).

Photoferrotrophs shuttle electrons from Fe(II) to the reaction center, suitably lowering the reduction potential of these electrons with energy from light for the reduction of carbon dioxide. Two mechanisms have been proposed for the purple non-sulfur bacteria; the Pio (phototrophic iron oxidation) system in *Rhodopseudomonas palustris* TIE-1, and Fox (Fe(II) oxidation) in *Rhodobacter ferrooxydans* SW2. The *pio* operon encodes three genes; *pioABC* (Jiao and Newman, 2007). Homologous to MtrAB in the Fe(III)-reducing system of *Shewanella*, PioA and PioB are thought to encode the ferroxidase and the integral outer membrane protein that anchors it in the membrane,

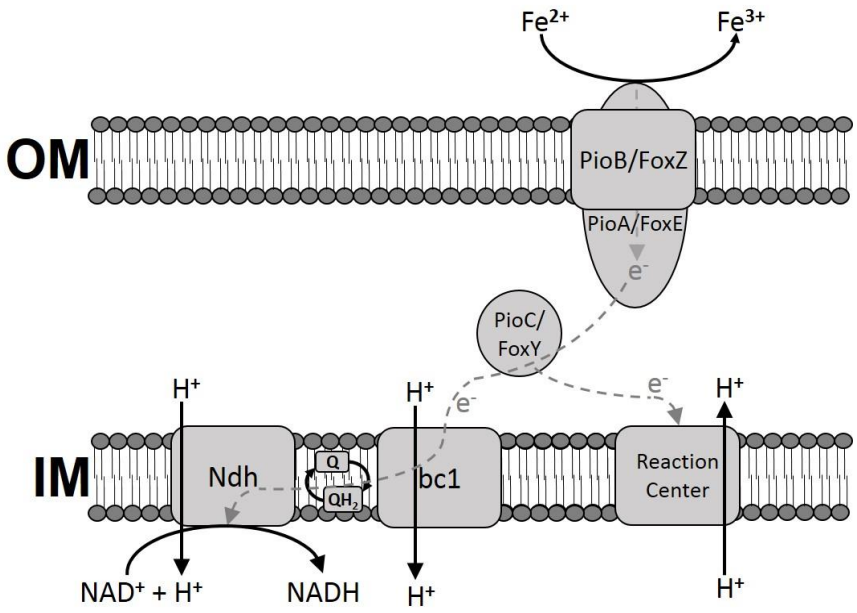

Figure 1.2. Model for Fe(II)-oxidation in *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter ferrooxydans* SW-2. Dotted lines follow proposed electron flow from Fe(II) to inner membrane components. In *Rhodopseudomonas palustris* TIE-1, it is proposed PioB anchors the decaheme cytochrome PioA in the outer membrane. PioA acts as the ferroxidase, though it remains unknown whether this occurs in the periplasm or extracellularly. Electrons are then likely passed to a periplasmic ferredoxin electron transport protein PioC. Depending on the cells energetic requirements, the electron may flow through reverse electron transport to a reducing equivalent, or be passed to the reaction center and used in photosynthesis. In *Rhodobacter ferrooxydans* SW-2, the diheme ferroxidase FoxE is likely anchored in the outer membrane by FoxZ. Electron from Fe(II) are passed from FoxE to FoxY, the periplasmic pyrrolopyrole quinone electron carrier. Depending on the cells energetic requirements, the electron may flow through reverse electron transport to a reducing equivalent, or be passed to the reaction center and used in photosynthesis. Abbreviations: OM, Outer Membrane; IM, Inner Membrane; Ndh, NADH Dehyrogenase; aa_3; Q, Ubiquinone; QH_2,

respectively (Jiao and Newman, 2007). PioC is thought to encode a soluble high-potential iron protein that acts as an electron shuttle through the periplasm (Jiao and Newman, 2007). In the three-gene *fox* operon, *foxE* is thought to encode the bihemic ferroxidase which transfers electrons to the periplasmic pyrrolopyrole quinone-based electron shuttle encoded by *foxY* (Croal et al., 2007). The final member of the *fox* operon, *foxZ* putatively encodes an inner membrane transporter, but has yet to be assigned a role in Fe(II) oxidation. The O_2/H_2O redox couple has one of the largest reduction potentials of the physiological electron acceptors; around +810 mV verses a standard hydrogen electrode at neutral pH. However, the rapid abiotic oxidation of Fe(II) by oxygen provides a considerable hurdle when metabolically coupling Fe(II) oxidation to the reduction of

molecular oxygen to take advantage of the potential. Because the rate of abiotic iron oxidation is dependent on the concentration of molecular oxygen, Fe(II) oxidizing bacteria rapidly become unable to compete as oxygen concentrations approach atmospheric levels. Microaerophilic Fe(II) oxidizing microbes take advantage of the decreased abiotic rates at lower oxygen concentrations, allowing the utilization of a high potential electron acceptor while remaining competitive with the abiotic rates. Occurring around neutral pH, microaerophilic Fe(II) oxidation results in the unavoidable production of large quantities of insoluble Fe(III) products. These precipitates are in stoichiometric abundance relative to biomass, and are difficult to separate from the organisms producing them, impeding biochemical and genetic analysis. Additional complication in the study of microaerophilic Fe(II) oxidizing metabolism arises from the known microaerophilic

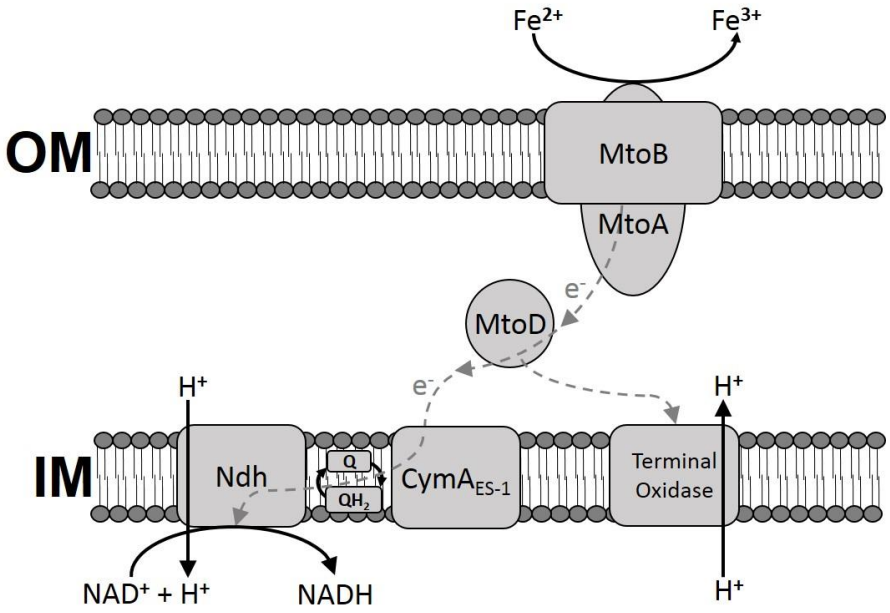

Figure 1.3. Model for Fe(II)-oxidation in *Sideroxydans lithotrophicus* ES-2. Dotted lines follow proposed electron flow from Fe(II) to the inner membrane. MtoB imbeds the decaheme ferroxidase MtoA in the outer membrane. Electrons are transported across the periplasm to the inner membrane tetraheme cytochrome $CymA_{ES-1}$ by the monoheme cytochrome MtoD. Abbreviations: OM, Outer Membrane; IM, Inner Membrane; Ndh, NADH Dehyrogenase; Q, Ubiquinone; QH_2, Ubiquinol.

Fe(II) oxidizing microbes being obligately ferrolithoautotrophic, or solely capable of utilizing iron and carbon dioxide for electrons and carbon.

While microaerophilic Fe(II) oxidizing organisms remain genetically intractable and thus the mechanism enigmatic, some clues as to the potential proteins involved can be determined from available sequence data. The genome of the freshwater Fe(II) oxidizing *Sideroxydans lithotrophicus* contains a homologue of the tetraheme CymA, an inner membrane electron carrier in *Shewanella* species. In *Shewanella*, CymA is involved in the extracellular reduction of Fe(III) (Schwalb et al., 2003), and thus may function in an reverse fashion in Fe(II) oxidizers. The genomic context of the gene encoding the CymA homologue ($CymA_{ES-1}$) is also promising, as it may be operonic with a decaheme cytochrome (*mtoA*), a putative outer membrane porin (*motB*), and a putative periplasmic monoheme cytochrome (*mtoD*); features commonly observed in transmembrane electron transport systems (Beckwith et al., 2015; Liu et al., 2012b). Exogenous expression of the decaheme cytochrome is able to partially restore Fe(III) reduction in an Δ*mtrA* *Shewanella oneidensis* strain, though the native role of this protein remains unclear a model for a potential mechanism has emerged (Figure 1.3)(Liu et al., 2012b). The decaheme cytochrome is not ubiquitously present among Fe(II) oxidizing microbes, suggesting either it does not function in Fe(II) oxidation, or there remains the possibility of multiple mechanisms. The genome of the marine Fe(II) oxidizer *Mariprofundus ferrooxydans* contains homologues to the Cyc2 ferroxidase of *Acidothiobacilus ferrooxydans*, though it remains unknown if this protein functions in a similar capacity (Ishii et al., 2015).

Fe(III) Reduction Systems

Microbial dissimilatory reduction of Fe(III) shares similar difficulties as Fe(II) oxidation, namely in the requirement to transfer electrons across insulating membranes due to an insoluble product (Figure 1.4). The demonstrated reversibility of the well described Fe(III)-reducing system of *Shewanella oneidensis,* and the use of these reverse-flow electrons to reduce intracellular products, provides a described system possibly capable of Fe(II) oxidation (Ross et al., 2011; Rowe et al., 2017). While *Shewanella* has yet to be demonstrated as capable of growing under these conditions, genetic comparison to model Fe(II) oxidizer genomes suggest homologues may exist between the metabolisms. In contrast to many of the model organisms for Fe(II) oxidation, several species of *Shewanella* are genetically tractable, enabling the study of an analogous system, as well as a suitable host for exogenous expression. The Mtr (metal reduction)

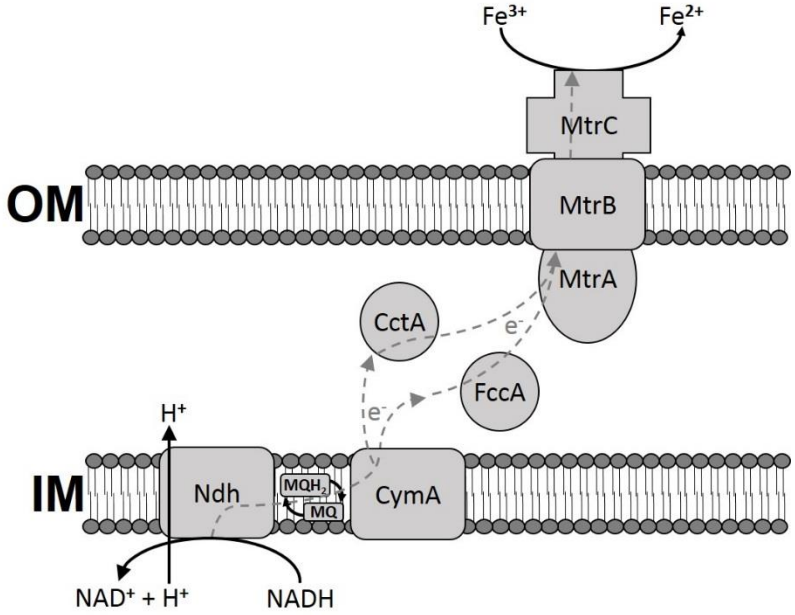

Figure 1.4. Model for Fe(III)-reduction in *Shewanella oneidensis* MR-1. Dotted lines follow electron flow from the inner membrane to Fe(III). Electrons originating from NADH are passed from NADH dehydrogenase to the inner membrane tetraheme cytochrome CymA via menaquinone. From CymA, electrons likely reach the decaheme cytochrome MtrA of the outer membrane complex through periplasmic carriers CctA and FccA. MtrB facilitates MtrA contact with the extracellular decaheme cytochrome MtrC, the terminal reductase. Abbreviations: OM, Outer Membrane; IM, Inner Membrane; Ndh, NADH Dehyrogenase; MQ, Menaquinone; MQH_2, Menaquinol.

system of *Shewanella* involves the tetraheme inner-membrane cytochrome CymA, which accepts electrons from the quinone pool originating from metabolism (Schwalb et al., 2003). These electrons are transferred to the periplasmic facing outer-membrane decaheme cytochrome MtrA, which is anchored in the membrane by the beta-barrel protein MtrB. MtrB facilitates transmembrane electronic contact of MtrA with the outer surface outer-membrane decaheme cytochrome MtrC. MtrC then acts as the ferric reductase, either directly or via soluble electron shuttles (Coursolle and Gralnick, 2010).

Electron transfer proteins

Central to extracellular electron transport are interacting proteins and small molecules capable of directionally accepting and donating electrons. The directionality is essential to maintaining cellular homeostasis, and is usually achieved by transferring electrons through a series of components of sequential reduction potentials. Components of proteins conferring electron transfer potential are known as redox centers, and are composed of either metals or small molecules. These redox active centers are usually prosthetic to proteins, where the local environment effects reduction potentials to fine tune a redox center to a particular function (Marshall et al, 2009). Particular arrangements of side groups facilitate the loading of a specific prosthetic, and thus can be predicted from sequence information if the motif is known and the residues are proximal in the primary sequence. Sulfur, sulfur-bearing side groups, and histidine are often used to coordinate redox centers employing transition metals to facilitate electron transfer such as cupridoxins, Fe-S proteins, and cytochromes. Common traits and motifs allow strait forward prediction of potentially redox active proteins from sequence data even when

described homologues are unavailable. Redox centers that mediate electron transfer via small molecules however, vary considerably. Conserved domains in proteins that utilize small molecule prosthetics such as flavins and quinones are better identified through phylogenetic comparison, as motifs are less universally conserved, making sequence-based analysis more difficult.

Chapter 2: *Marinobacter subterrani*, a Genetically Tractable Neutrophilic Fe(II)-Oxidizing Strain Isolated from the Soudan Iron Mine

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Bonis, B. M. and Gralnick, J. A. (2015) *Marinobacter subterrani*, a genetically tractable neutrophilic Fe(II)-oxidizing strain isolated from the Soudan Iron Mine. *Frontiers in Microbiology, 6*, 719. doi: 10.3389/fmicb.2015.00719

Summary

We report the isolation, characterization, and development of a robust genetic system for a halophilic, Fe(II)-oxidizing bacterium isolated from a vertical borehole originating 714 m below the surface located in the Soudan Iron Mine in northern Minnesota, USA. Sequence analysis of the 16S rRNA gene places the isolate in the genus *Marinobacter* of the Gamma*proteobacteria*. The genome of the isolate was sequenced using a combination of short- and long-read technologies resulting in two contigs representing a 4.4 Mbp genome. Using genomic information, we used a suicide vector for targeted deletion of specific flagellin genes, resulting in a motility-deficient mutant. The motility mutant was successfully complemented by expression of the deleted genes *in trans*. Random mutagenesis using a transposon was also achieved. Capable of heterotrophic growth, this isolate represents a microaerophilic Fe(II)-oxidizing species for which a system for both directed and random mutagenesis has been established. Analysis of 16S rDNA suggests *Marinobacter* represents a major taxon in the mine, and genetic interrogation of this genus may offer insight into the structure of deep subsurface communities as well as an additional tool for analyzing nutrient and element cycling in the subsurface ecosystem.

Introduction

Ubiquitous and abundant in marine environments, the genus *Marinobacter* contains a metabolic diversity and environmental versatility that has gained recent attention as a prospective biocatalyst for wax ester synthesis (Lenneman et al., 2013; Holtzapple and Schmidt-Dannert, 2007) and remediation of hydrocarbon contaminated

environments (Fathepure, 2014; Strong et al., 2013). *Marinobacter* species become initially enriched in hydraulic fracturing effluent, suggesting an ability to thrive in harsh environments and in the deep subsurface (Cluff et al., 2014). Coupled with the prominent contribution of *Marinobacter* to geochemical cycling (Handley and Lloyd, 2013; Wang et al., 2012; Handley et al., 2009) and various ecosystem roles such as hydrocarbon degradation (Handley and Lloyd, 2013) and marine snow precipitation (Kaeppel et al., 2012), a greater understanding and control of these bacteria is necessary to fully harness and explore the processes they influence and are able to perform. Though traditionally thought to be a genus comprised exclusively of marine organisms (Handley and Lloyd, 2013), *Marinobacter* species isolated from salinous non-marine sources are challenging this definition of the genus, which includes members from wastewater (Liebgott et al., 2006), salinous soil (Martin et al., 2003), and now the deep subsurface.

The Soudan Underground Mine State Park, home of the Soudan Iron Mine, is located in the Archean Soudan Iron Formation of northern Minnesota. The mine reaches a depth of 714 m below the surface, and produced high-grade hematite ore until it closed operations in 1962. In an effort to expand the depth of the mine prior to closing, core samples were drilled to locate ore-rich veins. Water from the surrounding rock, a calcium- and sodium-rich brine that reaches salinities as high as 4.2% (w/v), emerges from these boreholes. Anaerobic and containing up to 150 mM dissolved iron, the water carries with it microbes from still deeper in the earth (Edwards et al., 2006). Opposing concentration gradients of Fe(II) and oxygen form as the effluent contacts the air in the mineshaft, creating an environment conducive for aerobic microbial Fe(II) oxidation. A bacterium belonging to the genus *Marinobacter*, designated isolate JG233, was isolated

from a downwardly oriented borehole located on the lowest level of the mine, approximately 714 m below the surface. A previous study conducted by Edwards *et al.* in 2006 focusing on microbial ecology of the Soudan Iron Mine, found abundant Gammaproteobacteria in this level of the mine (Edwards et al., 2006). Heterotrophic cultivation estimates concentration of *Marinobacter* to be approximately 10^5 CFU / mL from the site where JG233 was isolated (data not shown). These findings are further supported by cultivation-independent analysis where *Marinobacter* species constituted a significant fraction of 16S rDNA sequences from several sites within the mine (unpublished data). The presence of *Marinobacter* suggests the genus is well suited to survival in the high salinity and Fe(II) found in the mine, and as such is likely to influence nutrient and geochemical cycling.

The quantity of iron in the earth's crust and its availability for electron transfer reactions enable it to significantly impact the cycling of other elements, and has been implicated in the change of state of carbon, sulfur, oxygen, nitrogen, and manganese (Johnson et al., 2012; Konhauser et al., 2011; Ghiorse, 1984). These elements play critical roles in the environment; further extending the already considerable influence of iron alone. The oxidation state of iron also influences soil structure, dissolved carbon stability (Chan et al., 2009), and enzyme activity (Baldock and Skjemstad, 2000; Bronick and Lal, 2005), affecting microbial communities and soil fertility. Additionally, microaerophilic Fe(II)-oxidizing bacteria have been implicated in the accelerated corrosion and occlusion of water-associated iron-bearing constructs, biofouling, and corroding pipes, as well as other iron-containing structures (Emerson et al., 2010; McBeth et al., 2010; Lee et al., 2013). In the environment, Fe(II)-oxidizing organisms

facilitate weathering and cycling, affecting the oxidation state, bioavailability, and solubility of a variety of important elements including carbon, oxygen, nitrogen, and sulfur. Despite the importance and prevalence of these organisms, little is known regarding the biochemistry of microaerophilic Fe(II) oxidation. To our knowledge there exists no genetically tractable representative of the Fe(II)-oxidizing bacteria, and only recently has genomic work become possible. This lack of knowledge arises from two complicating factors inherent to Fe(II)-oxidizing metabolisms: the quantities of Fe(III) precipitates that result as a product of Fe(II) oxidation are inhibitory to biochemical and genetic analysis, and it is difficult to obtain sufficient biomass for analysis. These problems are coupled in current model organisms for microaerophilic Fe(II) oxidation, as growth on Fe(II) is obligatory for these organisms. For this study we set out to isolate, characterize, and describe a model organism for the study of Fe(II) oxidation capable of heterotrophic growth, and to develop a robust genetic system for this model organism. Here we describe an environmental isolate that represents a microaerophilic Fe(II)-oxidizing strain capable of heterotrophic growth, uncoupling an increase in cell density from a proportional increase in Fe(III) byproducts. Heterotrophic growth allows for the use of established molecular techniques without the complications of growth on Fe(II) normally observed in Fe(II)-oxidizing model organisms.

Materials and Methods

Isolation

Samples were obtained from the effluent of a descending exploratory borehole, Soudan Mine Diamond Drill Hole 942, located in the bottom (level 27) of the Soudan

Underground Mine State Park in Soudan, Minnesota. The effluent is an iron-rich, primarily calcium chloride brine with a circumneutral pH (6.31±0.48). Strain JG233 was isolated aerobically on agar-solidified Difco Marine Broth (MB) medium (Becton Dickenson and Company, NJ).

Media and Growth Conditions

Escherichia coli cultures were grown at 37°C in Difco LB (Luria-Bertani) medium. When cultivated in rich medium, isolate strain JG233 was grown at 30°C in MB medium. In defined-media experiments, isolate strain JG233 was cultured at 30°C in *Marinobacter* Iron Medium (MIM) containing per liter: 50.0 g NaCl, 5.3 g $MgCl_2 \cdot 6H_2O$, 0.75g KCl, 0.1 g $MgSO_4 \cdot 6H_2O$, 50 mg K_2HPO_4, 1.0 g NH_4Cl, 0.740 g $CaCl_2 \cdot 2H_2O$, 0.42 g $NaHCO_3$, 2.38 g HEPES (4-(hydroxyethyl)-1-piperazineethanesulfonic acid) and pH adjusted to 7.0 with 1 M HCl. LBMB (Luria-Bertani Marine Broth) medium used during conjugation contained 750 mL prepared Difco LB and 250 mL prepared Difco MB per liter. MB25RB medium used in preparation of competent cells contained per liter: 3.75 g tryptone, 1.875 g yeast extract, 1.875 g NaCl, 1mL 1N NaOH, 4.39 g $MgSO_4 \cdot 6H_2O$, and 4.675 g Difco MB powder. For growth under kanamycin selection, media contained 50 µg/mL kanamycin sulfate (Fisher Scientific, MA). Diaminopimelic acid was used at a concentration of 360 µM when growing *E. coli* strain WM3064. Media were supplemented with 1.5 % (w/v) agar for growth on solid media.

Growth conditions were assayed aerobically in MIM at 30°C unless varied for the conditions of the assay as noted. Heterotrophic growth of isolate strain JG233 in liquid media was monitored using optical density at 600 nm, unless the opacity of the media

proved prohibitive, in which case growth was determined by counting colony forming unit (CFU) = on MB solid medium. Electron donors were assayed at 20 mM or 0.03% unless noted. Media for anerobic growth were prepared by sparging MIM with N_2 gas for 15 min in Balch tubes prior to autoclaving sterilization. Media were amended with 10 mM sodium lactate as the electron donor and 40 mM electron acceptor. Following growth in tubes containing nitrate, nitrogen speciation was determined using 0.8% N,N-dimethyl-α-napthylamine solubilized in 5 M acetic acid and 0.6% sulfanilic acid solubilized in 5 M acetic acid, followed by the addition of powdered zinc. Salinity assays utilized 40 mM sodium lactate as the electron donor and varied concentrations of NaCl and $CaCl_2$ in MIM media from 0 M to 2.22 M, and 0 mM to 300 mM, respectively. Hydrochloric acid and sodium hydroxide were used to adjust MIM for pH assays, and contained 40 mM sodium lactate as the electron donor. Media for assaying pH tolerance contained 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and N-cyclohexyl-2-aminoethanesulfonic acid (CHES) to extend buffering capacity. Antibiotic susceptibility was assayed using antibiotic disks AM10, N30, E15, TE30, P10, S10, and B10 (Cypress Diagnostics, Belgium) on solid LB medium.

Fe(II) Oxidation Assay

The gel-stabilized gradient tube system from Emerson and Floyd (2005) was adapted for Fe(II) oxidation assays (Emerson and Floyd, 2005). In brief, FeS or $FeCO_3$ stabilized in 1.0% agarose was deposited in the bottom of sterile Balch tubes and allowed to solidify. A low-melt agarose (SeaKem LE Agarose, Lonza, ME) stabilized MIM at pH 7.0 and lacking HEPES, was gently overlaid on the Fe(II)-plugs and gassed for 1 min

using 20% CO_2 gas with a balance of N_2. Tubes were then stoppered with butyl rubber bungs and allowed to set overnight at ambient temperature. Heterotrophically grown strain JG233 cultures were washed in defined medium lacking organic carbon before inoculation into the gradient tubes. Inoculation was performed by removing the bung from the tube, allowing atmospheric gas into the headspace, and injecting cells throughout the length of the tube. Gradient tubes were incubated statically in the dark at 30°C. During respiratory inhibition, gradient tubes of varied maturity were amended to a final concentration of 2 mM sodium azide or 20 µM N,N-dicyclohexylcarbodiimide (DCCD) solublized in 200 proof ethanol.

Genomic DNA isolation and genome assembly

For Illumina sequencing, genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega, WI) from an overnight culture of isolate strain JG233 grown in MB medium. Purified DNA was sequenced at the University of Minnesota Genomics Center using an Illumina platform sequencing 100 bp paired-end reads. For PacBio sequencing, pooled samples from 5 mL overnight outgrowths of isolate strain JG233 were isolated using phenol/chloroform extraction. Purified DNA was sent to the Mayo Clinic Bioinformatics Core for sequencing on a PacBio platform using size selected reads on 4 SMRT cells. Assembly was done using the Hierarchical Genome Assembly Process with 100X coverage. The genome was assembled to two contigs and polished to accuracy greater than 99.99%. Auto-annotation was performed using the RAST Annotation Server through the SEED <http://www.theseed.org/> (Aziz et al.,

2008) (Overbeek et al., 2014). G+C content was determined from the genome sequence and annotation report.

Bacterial Strains and Mutant Construction

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Primers were purchased from Integrated DNA Technologies. DNA modification enzymes were purchased from New England BioLabs. DNA purification for plasmid construction was achieved using the Invitrogen Quick PCR Cleanup Kit and Quick Plasmid Miniprep Kit.

For *flaBG* mutant construction, 1 kb regions flanking the *flaBG* genes from isolate strain JG233 were amplified by PCR in two reactions, one using primers Flagellin UF/Flagellin UR, and the other with Flagellin DF/Flagellin DR (Table 1) in GoTaq Green Master Mix (Promega, WI). Purified upstream and downstream amplicons were digested using either BamHI/AflII or AflII/SpeI, respectively. The vector, pSMV3 (Saltikov and Newman, 2003), was digested using BamHI/SpeI and gel purified. Fragments and vector were ligated using T4 ligase prior to transformation into *E. coli* strain UQ950.

Preparation of Competent Cells

Chemically competent isolate strain JG233 stocks were prepared using an adapted protocol for rubidium chloride treatment. Briefly, cultures were grown to an optical density at 600 nm of 0.75 in MB25RM medium then kept on ice for the remainder of the preparation. Cultures were centrifuged and pellets resuspended in TFB1 containing per 500 mL: 1.45 g potassium acetate, 6 g rubidium chloride, 0.75g $CaCl_2 \cdot 2H_2O$, 4.95 g

Table 2.1. Bacterial strains, vectors, and primers

Strain or vector	Relevant Trait(s)	Source
Strains		
Marinobacter subterrni		
JG233	Wild Type	This Study
JG2729	Δ*flaBG*	This Study
JG3126	Wild Type with pBBR1MCS-2	This Study
JG2756	Δ*flaBG* with pBBR1MCS-2 containing *flaBG*	This Study
JG2791	JG233 with pBBR1MCS-2 containing *gfp*mut3	This Study
Escherichia coli		
UQ950	DH5α vector construction host	1
WM3064	DAP auxotroph donor strain for conjugation	1
Vectors		
pSMV3	Deletion vector, Km^r, *sacB*	1
pSMV3Δ*fla*BG	Deletion construct for *flaBG*	This Study
pBBR1MCS-2	Broad range cloning vector, Km^r	2
pBBR1MCS-2::*fla*BG	Δ*flaBG* complementation vector	This Study
pBBR1MCS-2::*gfp*mut3	*gfp* containing vector	This Study
pMiniHimar RB1	Transposon-carrying plasmid	3
Primers		
Flagellin UF	catg**GGATCC**TTCTTCCTGTTTGGGACCGAC	This Study
Flagellin UR	catg**CTTAAG**GGCTAATGCCCCTCCAGTATC	This Study
Flagellin DF	cagt**CTTAAG**CAGTAAACTCAGAACGCCC	This Study
Flagellin DR	cagt**ACTAGT**GTGCCGGTTTCCTCGGAG	This Study
Flagellin CompF	cagt**AAGCTT**GATACTGGAGGGGCATTAGCC	This Study
Flagellin CompR	cagt**ACTAGT**GGGCGTTCTGAGTTTACTG	This Study
27F	AGA GTT TGA TCM TGG CTC AG	4
M13F	GTA AAA CGA CGG CCA GT	5
M13R	CAG GAA ACA GCT ATG AC	5

1. Saltikov and Newman (2003)
2. Kovach et al. (1995)
3. Bouhenni et al. (2005)
4. Wilson et al. (1990)
5. "Universal Primer List" (n.d.)

$MnCl_2 \cdot 4H_2O$, 94.5 g glycerol, and pH adjusted to 5.8 with acetic acid. Again, cells were centrifuged and pellets resuspended in TFB2 containing per 100 mL: 0.2 g MOPS, 1.1 g $CaCl_2 \cdot 2H_2O$, 0.12 g rubidium chloride, and 18.9 g glycerol, and pH adjusted to 6.5 with KOH. Stocks were stored at -80°C until use. For transformation of competent isolate strain JG233, aliquots were incubated with 120 ng pBBR1MCS-2::*gfp*mut3 for 30 min on ice. Following heat shock at 45°C for 5 min, cells were incubated for an additional 2 min on ice before dilution in MB25RB. After a 60 min recovery at 30°C, cells were plated to MB plates and MB plates supplemented with kanamycin.

Conjugation

E. coli donor strain WM3064 was used to conjugate plasmids into isolate strain JG233. Cultures of donor and recipient were grown in LB amended with kanamycin and Marine Broth, respectively, and then diluted to an optical density at 600nm of 1.0 with LBMB. Donor cultures were washed once with LBMB to remove kanamycin, pelleted, and decanted. After recipient cells were heat shocked at 45°C for 5 min, 1 mL was removed and used to resuspend the donor pellets. Cultures were pelleted, resuspended in 100 µL LBMB, and spotted onto LBMB plates. Following an 18 hour incubation at 30°C, spots were resuspended in LBMB and spread to MB plates devoid of diaminopimelic acid and amended with kanamycin for selection against the donor strain and for the selection of meridiploid mutants, respectively. Kanamycin resistant colonies were verified by polymerase chain reaction using insertion-specific primers. Selection on MB plates amended with 100 mM sucrose yielded kanamycin-sensitive colonies. Mutants were confirmed via Sanger sequencing of the 16S rRNA gene using 27F and genomic DNA using Flagellin UF and Flagellin DR primers to assure no contamination had occurred.

Phylogeny

Phylogenetic analysis was done using the two identical complete 16S rRNA genes of isolate strain JG233 obtained from the genome assembly. All sequences were size-adjusted, aligned, and a tree was constructed using the MEGA6.06 software package (Tamura et al., 2013) with the Neighbor-Joining statistical method with a Jukes-Cantor substitution model and 1000 bootstrap replications (Jukes and Cantor, 1969). Analysis of

gyrB was conducted similarly to the 16S rRNA genes using the MEGA6.06 software package (Tamura et al., 2013) utilizing the Maximum Likelihood method and the Tamura-Nei model with 1000 bootstrap replications (Tamura and Nei, 1993). Genome comparison was conducted using the Average Nucleotide Identity calculator developed by the Konstantinidis Lab, Georgia Institute of Technology, GA (Goris et al., 2007).

Results

Growth Conditions

Growth in MIM was supported by acetate, citrate, fumarate, fructose, glucose, glutamine, glycerol, lactate, n-hexane, n-tetradecane, succinate, and sucrose. No growth was observed in defined media containing arabinose, galactose, glucosamine, lactose, malate, or N-acetylglucosamine as the sole electron donor. Anaerobic oxidation of lactate was coupled to the reduction of nitrate to nitrite. Anaerobic growth was not supported by nitrite, sulfate, fumarate, DMSO, or TMAO, and no growth was observed in controls lacking a terminal electron acceptor. Isolate strain JG233 grew at temperatures between 2°C and 37°C, and between pH 5 to 9, with optimum growth rates at 37°C and pH 6. Strain JG233 is capable of growth in NaCl concentrations of 0.5-13% with an optimum at 7%. Isolate strain JG233 displayed zones of clearing around diffusion discs containing ampicillin, neomycin, erythromycin, tetracycline, penicillin, and streptomycin and no apparent growth defect from bacitracin.

Genome Properties

The draft genome of strain JG233 consists of two contigs of a single amplicon, encompassing 4,453,613 bp, 4,155 possible coding regions, and a G+C content of 58.9%. The finished assembly had an N_{50} of 3,410,286 and a length cutoff of 15,849 bp. Despite read lengths up to 40kb, the assembly was unable to resolve two repetitive regions containing phage sequences of high similarity. Analysis of the SEED metabolic reconstruction using the KEGG database supports strain JG233 aprototrophic for amino acid biosynthesis, which is empirically supported by growth in defined media. Analysis of the genome also suggests complete glycolysis, Entner-Doudoroff, pentose phosphate, and tricarboxylic acid cycle pathways. Carbon source utilization is supported by the genomic annotation. Based on genomic analysis, strain JG233 should be capable of fermentation, though cultivation-based efforts have failed to demonstrate this metabolism.

In an effort to identify gene products that may be involved in Fe(II) oxidation, sequences of proteins thought to be involved with Fe(II) oxidation in other organisms were queried against the strain JG233 translated genome. Though a putative protein with 32% identity to the CycA1 of the acidophilic Fe(II)-oxidizer *Acidothiobacillus ferrooxidans* was identified, the lack of genes encoding Cyc2 or Rusticyanin fails to suggest that a similar Fe(II) oxidation system is employed by strain JG233. Strain JG233 lacks homologs to proposed photosynthetic Fe(II) oxidation systems PioABC (Jiao and Newman, 2007) and FoxEYZ (Croal et al, 2007) from *Rhodopseudomonas palustris* TIE-1 and *Rhodobacter ferrooxidans* SW-2, respectively, as well as the proposed MtoAB and $CymA_{ES\text{-}1}$ (Liu et al, 2012) of the neutrophilic Fe(II)-oxidizer *Sideroxydans lithotrophicus*

ES-1. PioAB and MtoAB share high sequence identity with MtrAB of the Fe(III)-reducing system of *Shewanella oneidensis* MR-1. Homology between these systems suggests other genes involved with Fe(III) reduction may share orthologs in Fe(II)-oxidizing organisms, and the genome of JG233 was queried and found to be lacking homologs to MtrABCDEF, DmsEF, OmcA, and the translated products of SO_4359 and SO_4360 of *Shewanella oneidensis* MR-1. The relevance of *c*-type cytochromes in electron transfer systems was also exploited to identify candidate genes for Fe(II) oxidation. The translated genome of strain JG233 was searched for proteins containing the characteristic *c*-type cytochrome binding CXXCH motif (Kranz et al, 2009), yielding 33 possible *c*-type cytochromes. Of these, four contained two heme *c*-binding motifs, and none more than two.

Phylogeny

Analysis of the 16S rRNA gene of isolate JG233 places it in the genus *Marinobacter*, however, members of the genus *Marinobacter* often share a greater than 97% identity of the 16S rRNA gene (Figure 2.1). Few of the *Marinobacter* have additional publicly available sequences, making further sequence-based analysis difficult. Taxonomic classification of isolate JG233 was refined though comparison of gene *gyrB*, average nucleotide identity, and phenotypic properties. The *gyrB* gene is commonly used

as an indicator of phylogenetic relatedness, as it contains sufficient length to tolerate subtle mutations that accumulate during speciation, but remains a highly conserved supplement or alternative to classification based on the 16S rRNA genes (Rajendhran and Gunasekaran, 2011). Establishment of strain JG233 as a distinct species from *M. adhaerens*, *M. salarius*, *M. similis*, and *M. flavimaris* is supported by comparison of

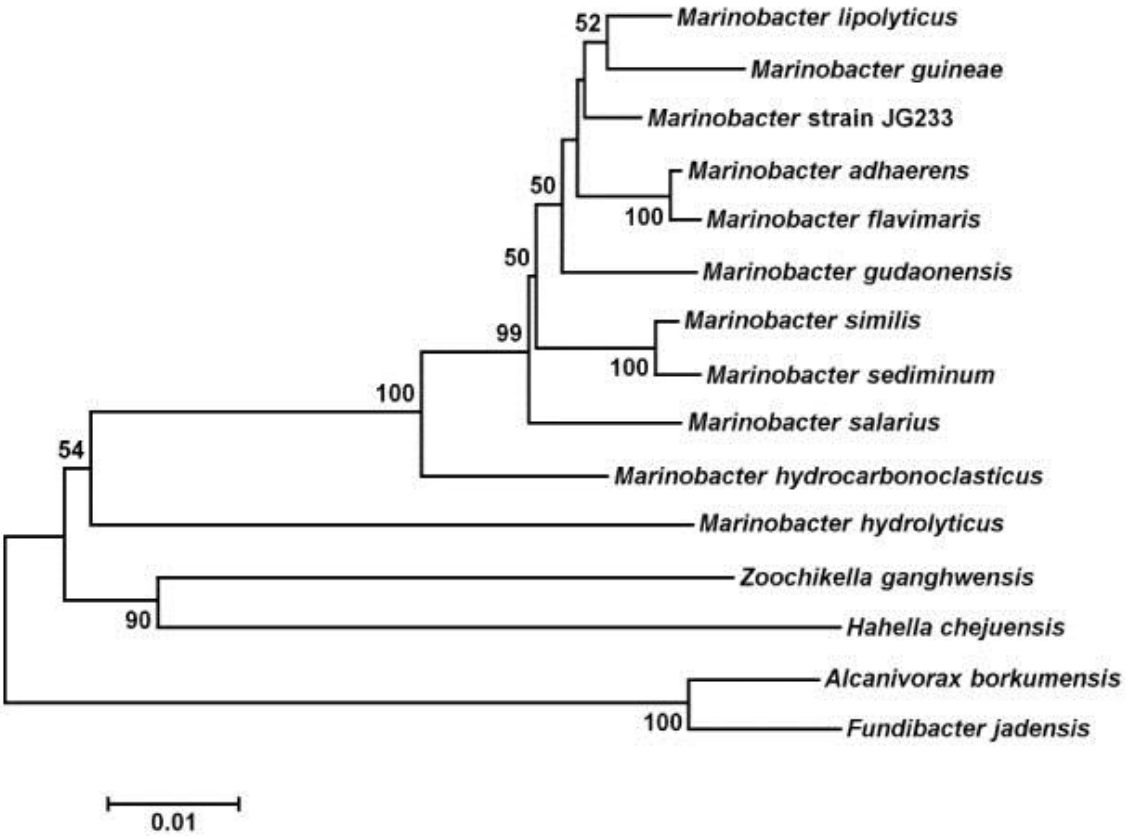


Figure 2.1. Maximum Likelihood phylogenetic tree of the 16S rRNA gene placing Marinobacter subterrani JG233 in the genus Marinobacter. Evolutionary relatedness was inferred using the Neighbor-Joining method based on the Jukes-Cantor method (Jukes and Cantor, 1969). Branch lengths are measured in the number of substitutions per site. Sequences were obtained from the National Center for Biotechnology Information, and were trimmed to as to attain a total of 1417 positions in the final dataset representing similar regions of the 16S rRNA gene. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Accession numbers for included species are as follows: Marinobacter adhaerens (NR_074765), Marinobacter adhaerens (NR_074765), Marinobacter similis (KJ547704), Marinobacter gudaonensis (NR_043796), Marinobacter salarius (KJ547705), Marinobacter lypolyticus (NR_025671), Marinobacter flavimaris (NR_025799), Marinobacter sediminum (NR_029028), Marinobacter guineae (NR_042618), Marinobacter hydrocarbonoclasticus (NR_074619), Zooshikella ganghwensis (AY130994), Microbulbifer hydrolyticus (AJ608704), Alcanivorax borkumensis (Y12579),Fundibacter jadensis (AJ001150), and Hahella chejuensis (AF195410).

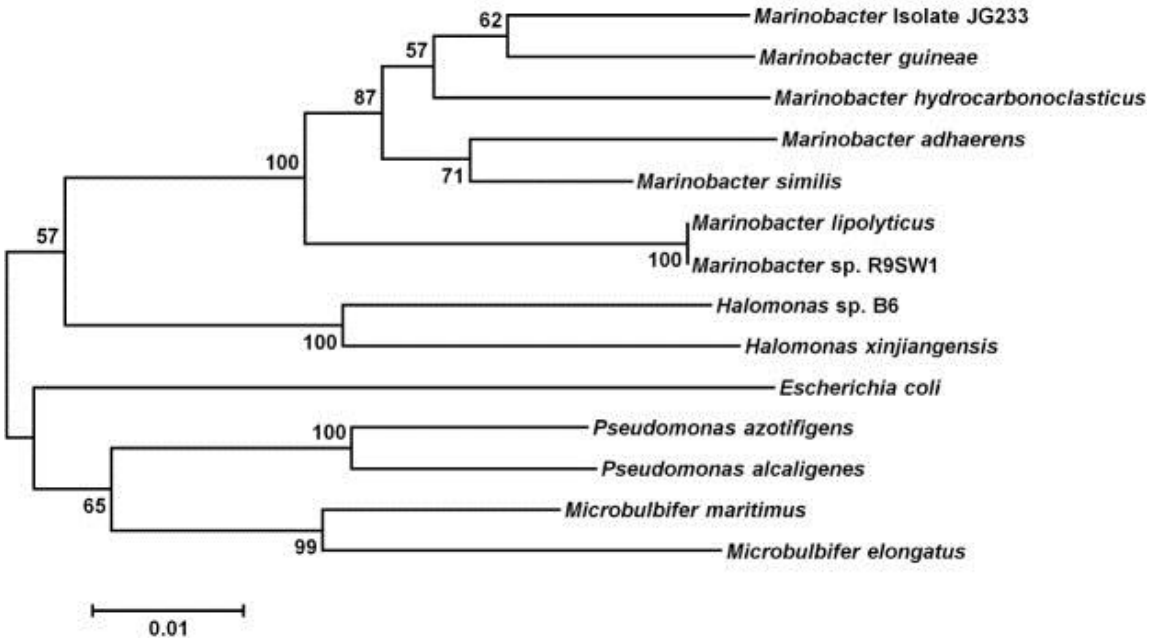


Figure 2.2. Maximum Likelihood phylogenetic tree of the *gyrB* gene. Evolutionary relatedness was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Branch lengths are measured in the number of substitutions per site. Sequences were obtained from the National Center for Biotechnology Information, and were trimmed so as to attain a total of 851positions in the final dataset representing similar regions of the *gyrB* as constrained by sequence available from the National Center for Biotechnology Information. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). Accession numbers for included species are as follows: *Marinobacter adhaerens* (KF811467), *Marinobacter salarius* (KJ547705), *Marinobacter similis* (CP007151), *Marinobacter guinea* (KJ467768), *Marinobacter* sp. R9SW1 (KF811464), *Marinobacter hydrocarbonoclasticus* (KF811470), *Halomonas sp. B6* (KC935335), *Halomonas xinjiangensis* (KC967623), *Pseudomonas azotifigens* (KM103930), *Pseudomonas alcaligenes* (AB039388), *Microbulbifer maritimus* (AB243198), *Microbulbifer elongatus* (AB243199), E*scherichia coli* (AB083821).

gyrB, which clusters isolate JG233 solely, but distinctly, with *M. guineae* (Figure 2.2). Genomes publicly available for species with an identity higher than 97% to the 16S RNA gene of isolate JG233 include *M. adhaerens*, *M. salarius*, *M. similis, and M. lypoliticus*. Two-way average nucleotide identity comparison to the strain JG233 genome yield values of 84.73%, 79.51%, 80.15%, and 77.23% respectively. These values fall conservatively outside the 95% threshold put forth by Goris, *et al.* (2007) for speciation.

Genetic System

Conjugation of the pMiniHimar RB1 transposon vector into wild-type isolate strain JG233 resulted in kanamycin-resistant colonies. Presence of the transposon was confirmed by sequencing purified genomic DNA using transposon specific primers (data not shown). Direct transformation of isolate strain JG233 with plasmid pBBR1MCS-2::*gfp*mut3 was achieved by chemically induced competency with an efficiency of 1.36 x 10^4 CFU/µg plasmid DNA. Directed deletion of the chromosomal region containing flagellin encoding genes *flaG* and three copies of *flaB* was conducted via conjugation of the suicide vector pSMV3Δ*flaBG* into wild type isolate strain JG233, followed by sucrose counter selection. Flagellin deletion mutants were confirmed by Sanger sequencing and a lack of motility in 0.3% agar MB plates (Figure 2.3). Motility was recovered upon conjugation of the pBBR1MCS-2::*flaBG* complementation construct into motility-deficient mutants (Figure 2.3c), and motility was not observed by the pBBR1MCS-2 empty-vector controls (data not shown).

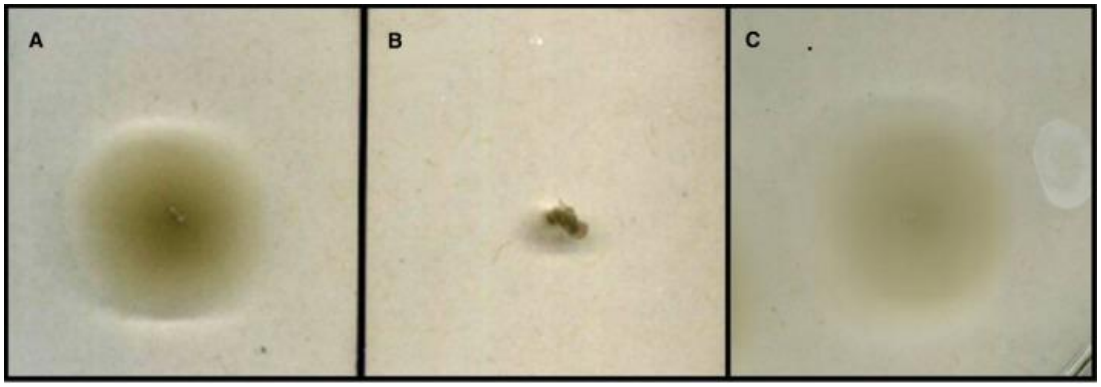


Figure 2.3. Swimming motility assay. Comparison of wild type *Marinobacter* strain JG233 (A) with Δ*flaBG* (B) and the Δ*flaBG* mutant complemented with pBBR1-MCS2:: *flaBG* (C) on 0.3% agar Marine Broth plates.

Fe(II) Oxidation

Isolate strain JG233 was tested for Fe(II) oxidation in gradient tubes and positively demonstrated Fe(II) oxidation with either FeS (Figure 2.4) or $FeCO_3$ (results not shown) as the Fe(II) source as compared to abiotic and *E. coli* UQ950 controls. Growth was not observed in gradient tubes when compared to controls lacking Fe(II). Use of respiratory inhibitors sodium azide and N,N-dicyclohexylcarbodiimide abolished the distinct band of Fe(II)-oxides associated with microbially facilitated Fe(II) oxidation, resulting in banding consistent with abiotic Fe(II) oxidation(Figure 2.5). Ethanol was found to have no effect on the system in the volumes used to solubilize N,N-dicyclohexylcarbodiimide (results not shown). Scanning electron microscopy suggests cells do not form biogenically templated Fe(III) structures such as stalks or sheaths (results not shown).

Discussion

Prior to the cessation of mining operations in 1962, expansion of the Soudan Iron Mine was actively underway and involved the drilling of cores to survey the surrounding rock. Shortly after, anaerobic and Fe(II)-rich water started welling from the deeper of these boreholes. The effluent of these boreholes is ideal for microaerophilic Fe(II)-oxidizing bacteria as oxygen diffusion forms opposing Fe(II) and oxygen gradients.

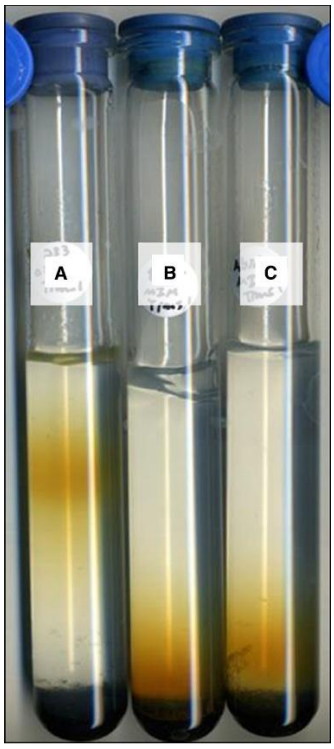


Figure 2.4. Fe(II) oxidation by *Marinobacter* strain JG233 in gradient tubes. Representative set of tubes (n = 3) demonstrating Fe(II) oxidation by *Marinobacter* strain JG233 (A) compared to *Escherichia coli* strain UQ950 (B) and an abiotic control (C) in tubes containing FeS as the Fe(II) source and MIM +0.15% low melt agarose as the medium overlay.

Figure 2.5. Fe(II) oxidation under respiratory inhibition. Representative set of *Marinobacter* strain JG233 in tubes containing FeS as the Fe(II) source and MIM +0.15% low melt agarose as the medium overlay. Results were consistent regardless of incubation time prior to respiratory inhibition, as long as inhibitor was added prior to rust-colored band formation. Abiotic **(A)** and with *Marinobacter* strain JG233 **(B)** both contain sodium azide to 2 mM. N,N-dicyclohexylcarbodiimide solublized in 200 proof ethanol was added to a final concentration of 20 µM to an abiotic control **(C)** and a tube with *Marinobacter* strain JG233 **(D)**. The tubes containing respiratory inhibitors exhibited rust-colored bands dissimilarly to uninhibited *Marinobacter* strain JG233 **(E)** and are located in similar positions as the abiotic control **(F)**, indicating Fe(II) oxidation by *Marinobacter* strain JG233 required actively metabolizing cells.

Isolated from one such vertical borehole originating 714 m below the surface and almost 1,600 km from an ocean, isolate JG233 attests to the ubiquity of the genus *Marinobacter*, a genus thought to primarily consist of marine organisms. *Marinobacter* constitutes a dominant genus in the mine, with representation in some mine samples greater than 70% based on 16S rDNA sequencing (unpublished data).

Description of Marinobacter subterrani sp. nov.

Marinobacter subterrani (suhb.tuh.reyn.ahy. Genitive noun *subterrani* meaning 'of the subterrane,' referring to the ecosystem from which the strain was isolated). Cells are motile rods approximately 1.8-2.0 µm in length and 0.4 µm wide under Fe(II) oxidizing conditions and 1.5 µm in length and 0.7 µm wide when grown under heterotrophic conditions. Colonies on MB agar are smooth, convex, circular, and have a diameter of approximately 1-2 mm after 48 hr of growth at 30°C. Growth occurs from pH 5-9 with an optimum around 6. Strain is moderately halophilic and capable of growth between NaCl concentrations of 0.5-13% (w/v) with an optimum at 7% (w/v). Interestingly, calcium is required for growth, with an optimum concentration of 0.54% (w/v) and a maximum tolerated concentration of 3.5% (w/v), though strontium can substitute for calcium to support growth. Divalent calcium is a major cation in the mine, reaching concentrations of 1.8% (w/v) at the sampling site.

The similarity to mine conditions of *Marinobacter subterrani* JG233 optima for salinity and pH, tolerance to calcium, and growth at 11^{o}C, coupled with the prevalence of *Marinobacter* in the mine, suggest *M. subterrani* is not only able to propagate but is well acclimated to the conditions from which it was isolated and has not recently been introduced to this environment. Despite significant 16S rDNA similarity to other members of the *Marinobacter;* average nucleotide identity, comparison of *gyr*B, distinct environmental niche, and physiological differences support the establishment of *M. subterrani* as a distinct species (Table 2).

The genome of *M. subterrani* does not contain components of any known carbon fixation pathways, and lithoautotrophic growth is neither expected nor observed (data not

shown). The lack of autotrophic growth with Fe(II) as an electron donor presents a challenge to determine the role of Fe(II) oxidation by this organism, as growth in the absence of alternative electron donors normally implicates the electrons from Fe(II) oxidation are used electrogenically. The inhibition of facilitated Fe(II) oxidation by respiratory inhibitors supports Fe(III)-precipitation by *M. subterrani* requires actively metabolizing cells, and thus is not thought to occur due to an unmediated process, such as production of acidic exopolysaccharides (Chan et al., 2009), which would persist regardless of viability. Regardless of mechanism or function of Fe(II) oxidation by *M. subterrani*, the prevalence of this organism, and the abundance of *Marinobacter* species isolated from cathodic enrichments from other oxic-anoxic interface environments (Wang et al., 2015; Rowe et al., 2015), imply this genus plays a major role in the communities that dominate Fe(II)-oxidizing environments.

Current models for the study of microaerophilic Fe(II) oxidation lack the necessary genetic tools to properly interrogate the possible mechanisms responsible for Fe(II) oxidation. This lack of a robust genetic system stems from the obligate nature of these model organisms for ferrotrophy, inherently coupling the growth of the organism to be studied to the production of Fe(III) oxides. The ability to grow *M. subterrani* under heterotrophic conditions enables the use of well-established methods for genetic manipulation separately from phenotypic observations when incubated with Fe(II). The establishment of a robust genetic system in *M. subterrani* has application beyond the study of Fe(II) oxidation. Phylogenetic analysis using16S rRNA gene would suggest

Table 2.2 *Marinobacter subterrani* JG233 and defining species attributes of related species.

Characteristics	1	2	3	4	5	6	7
DNA G+C Content (mol%)	52.7	57.1	58.9	56.9	58.0	57.6	57.1
Salinity Range (%[a] of Na^+)	0.5-20.5	1-15	1-13	0.5-20	20	0.5-20	0.5-20
Temperature Range (C°)	10-45	4-42	2-37	4-45	4-45	4-40	4-40
pH Range	6-9.5	5-9.5	5-9	5.5-10	5.5-8.0	6-9	6-9
Plasmids	2 (0.2Mb)		0	2		0	0
Utilization of:							
Acetate	+		+			-	+
Citrate	+	-	+	-		-	-
Fumarate	+		+				
Lactate			+	+		+	+
Malate			-	+			
Succinate	+		+			-	+
Arabanose	-		-	-	-	-	-
Fructose	-	+	+		+	-	+
Glucose	-	+	+	-	-	-	-
Glycerol	-	+	+	-	-	-	+
Lactose	-		-		-	-	-
Mannitol		-		-	-	-	-
NAG	-	+	-	-		-	-
Starch		+					
Sucrose	-		+	-	-	-	-
Hydrocarbons	+		+				
Reduction of nitrate	+	+	+	-	+	+	-
Reduction of nitrite	+	+	-		-	-	-
Fermentative		+	-				

[a] in weight per volume
NAG, N-acetyl-glucosamine
Spaces left blank were not assayed in the original characterization paper of the species
1, *M. hydrocarbonoclasticus* (Gauthier et al., 1992); 2, *M. guinea* (Montes et al., 2008); 3, *Marinobacter subterrani* JG233; 4, *M. adhaerens* (Kaeppel et al., 2012); 5, *M. flavimaris* (Yoon et al., 2004); 6, *M. similis* (Hooi et al., 2014); 7, *M. salarius* (Hooi et al., 2004)

inclusion of *M. subterrani* in the genus *Marinobacter* of the Gammap*roteobacteria*. *Marinobacter* are well regarded as a metabolically diverse clade, containing representatives capable of many biotechnologically and industrially relevant biochemical processes. The development of a genetic system for *M. subterrani* JG233 expands upon the genetic system for *M. adhaerens* reported by Sonnenschein et al. (2011) for the analysis and development of the genus *Marinobacter* and establishes a crucial genetic system for the study of microaerophilic Fe(II)-oxidizing species (Sonnenschein et al., 2011). The genetic tools outlined in this study allow methods previously unavailable in a microaerophilic Fe(II)-oxidizing strain to be utilized. Transposon mutagenesis,

markerless deletion, and complementation via expression from a plasmid enable the interrogation of the function of specific gene products, as well as the use of hypothesis generating experiments.

The isolation, description, and development of a genetic system in *M. subterrani* JG233 establishes a foundation for the genetic study of dominant organisms contributing to Fe(II) oxidation in the subsurface. As *Marinobacter* species are increasingly found in diverse environments, including hydraulic fracturing effluent, deep marine sediments, and beneath the iron ranges of Minnesota, a greater understanding of these microbes will shed light on survival and metabolism in the dark biosphere. Future studies should focus on the mechanism of the demonstrated Fe(II) oxidation and the contribution and mode of *M. subterrani* metabolism to the microbial community structure and its functioning in the native environment.

Acknowledgements

We would like to thank Daniel Bond and Jon Badalamenti for their diligence in the refinement and completion of the *M. subterrani* genome. We also thank Michael Donald and Ryan Lesniewski who helped initially characterize this strain while undergraduates in the Gralnick Lab. Funding for this research comes from the Minnesota Environment and Natural Resources Trust Fund.

Chapter 3: Fe(II) Oxidation in *M. subterrani*

Summary

The genetic tractability and ease of cultivation of *M. subterrani* enables unprecedented approaches to probe the underlying mechanism of microaerophilic Fe(II) oxidation. The elucidation of analogous systems in the acidophilic and photosynthetic Fe(II) oxidizers provides a genomic approach to survey the *M. subterrani* genome for gene products potentially involved in Fe(II) oxidation, and verify function using gene deletion and complementation. Fe(II) oxidation-independent growth allows cultivation dependent biochemical analysis. Observing growth, Fe(II) oxidation rates and distribution, and characteristic parameters allowing Fe(II) oxidation during gradient tube cultivation constrains the possible mechanisms involved in Fe(II) oxidation in *M. subterrani*.

Introduction

Mechanisms of Fe(II) oxidation

Microbes can facilitate Fe(II) oxidation via a variety of possible mechanisms that can be grouped into three general categories; metabolic, active, and passive (Summarized in Figure 3.1). In metabolic, the electrons from Fe(II) are used for the production of reducing equivalents or maintenance of proton motive force before being discarded to a terminal electron acceptor (Figure 3.1a). During autotrophic growth, these electrons can be utilized to reduce carbon for biomass. Under conditions where Fe(II) is the sole electron donor, growth is an adequate proxy to identify an organism as a metabolic Fe(II) oxidizer. *M. subterrani* lacks the capacity for autotrophic growth, thus requiring an alternative demonstration to discern electron utilization for chemolithoorganotrophy.

Arguments could be made for alternative proxies, such as demonstrating growth advantage, relative increase in cellular adenosine triphosphate (ATP), increased longevity, or continued motility in the presence of Fe(II).

Active Fe(II) oxidizing microbes do not gain a significant metabolic advantage from Fe(II) oxidation, but rather Fe(II) oxidation is mediated via products that are consumed during a reaction with Fe(II), and thus must be regenerated to allow the process to continue (Figure 3.1b). For example, active Fe(II) oxidation is inherent to nitrate-reducing organisms, as ferro-reactive nitrite and nitrous oxide products are produced (Carlson et al., 2013; Klueglein and Kappler, 2012). Other products of cellular activity capable of abiotic Fe(II) oxidation are generated during the oxidation of ammonia and sulfur, as well as extracellular superoxide produced by a variety of marine bacteria (Diaz et al., 2013). Note while there may not be a metabolic advantage directly from active Fe(II) oxidation, cells may facilitate this process as a mechanism to detoxify Fe(II) (Carlson et al., 2013; Poulain and Newman, 2009) or alter organic carbon to be more bioavailable (Caiazza et al., 2007).

Finally, a passive mechanism would involve a component of the cell or extracellular matrix that, once produced, is capable of catalyzing Fe(II) oxidation independent of the viability of the cell (Figure 3.1c). Previous work described in Bonis and Gralnick, 2015, using the respiratory inhibitors sodium azide and N,N-dicyclohexylcarbodiimide, suggest the Fe(II) oxidative phenotype of *M. subterrani* does not employ a passive mechanism, as inhibition halted Fe(II) oxidation (Bonis and Gralnick, 2015).

Fe(II) and oxygen gradient tubes

The gel stabilized gradient tube system first described by Emerson and Floyd is often employed for the cultivation of unicellular microaerophilic Fe(II) oxidizing microbes (Emerson and Floyd, 2005). In this system, opposing gradients of oxygen and Fe(II) are established, thus providing a multitude of oxygen to Fe(II) ratios for microaerophilic Fe(II) oxidizing microbes. A distinct band of Fe(III) precipitates form at the strata where conditions allow microbial Fe(II) oxidation, as opposed to a more diffuse band in abiotic controls. Conditional studies of the gradient tube system and the factors that perturb wild type Fe(II) oxidation provide a means to determine the nature of the mechanism involved in *M. subterrani* mediated Fe(II) oxidation. The mutual effects of

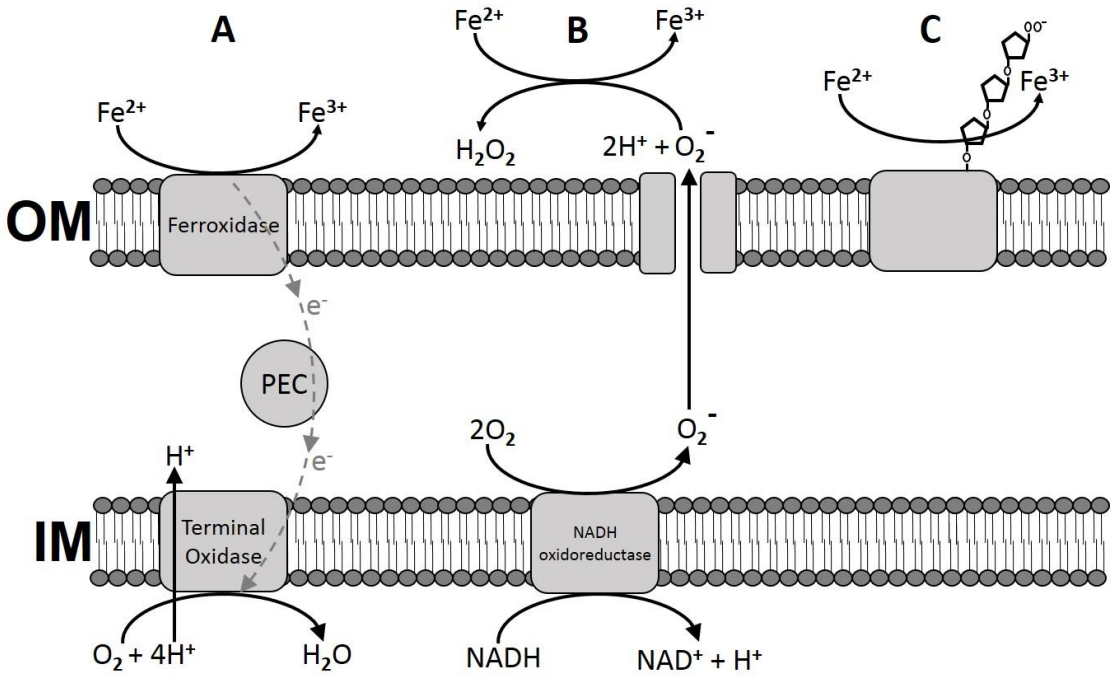


Figure 3.1. Potential mechanisms for Fe(II) oxidation. **A**. Example of a metabolic Fe(II) oxidation model. In this example, the electron removed from Fe(II) is transported to a terminal oxidase coupling site. **B**. Example of an active Fe(II) oxidation model. The reactant is produced by the cell and consumed during the reaction with Fe(II). **C**. Example of passive Fe(II) oxidation. In this example, an extracellular exposed glycoprotein acts as a catalyst for Fe(II) oxidation. Abbreviations: OM, Outer Membrane; IM, Inner Membrane; PEC, Periplasmic Electron Carrier.

Fe(II) on the growth and viability of *M. subterrani* and influences on Fe(II) oxidation facilitated by the organism, as well as factors including oxidation rate and locale are assayed.

Candidate genes for metabolic Fe(II) oxidation

In addition to conditional studies of Fe(II) oxidation, genomic and genetic approaches are also available in *M. subterrani*. Several gene clusters in the genome stand out with potential to encode systems involved in the Fe(II) oxidative phenotype, either displaying similarity to proposed electron transport systems, or containing motifs consistent with electron transport. The *pio*, *fox*, *mto*, and *mtr* operons contain several electron-carrying genes in proximity to a porin-like protein, forming a potential porin cytochrome complex (He et al., 2017). While the genome of *M. subterrani* does not contain homologues to any of the proposed Fe(II) oxidation systems, the putative diheme cytochrome encoded by Msub_12038 is in a favorable genomic context to suggest a transmembrane electron transport system. Proximal and similarly oriented, Msub_12038 is flanked by a putative ZIP metal transporter, Methyl accepting chemotaxis protein, NADH:flavin oxidoreductase, and a monoheme cytochrome. Given the model structure for the porin cytochrome complexes this potential operon resembles, deletion of Msub_12038 and the proximal monoheme cytochrome Msub_12037 should be adequate to abolish function.

Iron is essential to most life, though it is bioavailable only as aqueous Fe(III) and has very low solubility at neutral pH. An alternative to the production of chelators to obtain soluble Fe(III) is to oxidize soluble Fe(II) directly at the cell surface. A

multicopper oxidase, Mco, has been shown to facilitate assimilatory Fe(II) oxidation in *Pseudomonas aeruginosa,* achieving a specific activity of 0.13 μmol mg Mco^{-1} min^{-1} *in vitro* (Huston et al., 2002). He et al. suggested multicopper oxidases may be capable of acting as ferroxidases in dissimilatory Fe(II) oxidation as well (He et al., 2017). The genome of *M. subterrani* contains the putative multicopper oxidase Msub_12632. Genomic context of Msub_12632 suggests it may be involved with Fe(II) oxidation as it is proximal and possibly co-transcribed with metal tolerance and transport proteins.

Candidate mechanisms for active Fe(II) oxidation

Sulfur oxidation proceeds through a multistep process facilitated by the Sox proteins and evolves a variety of oxidized sulfur intermediates, several of which are capable of facilitating abiotic Fe(II) oxidation (Giles and Jacob, 2002; Mishanina et al., 2015). The *M. subterrani* genome contains the putative *soxHGFBAZYXDCH* operon (Msub_10588-Msub_10600), suggesting the organism may be capable of oxidizing sulfide as an electron donor. As the gradient tube system used in this study utilizes ferrous sulfide as an Fe(II) source, it is possible *M. subterrani* produces reactive sulfur species that abiotically react with Fe(II), as well as acting as an alternative electron donor.

Ammonia monooxygenase catalyzes the first step of ammonia oxidation where ammonia is oxidized to hydroxylamine, which is further oxidized to nitrite and nitrous oxide by hydroxylamine oxidoreductase (Caranto and Lancaster, 2017). Nitrite and nitrous oxide react abiotically to oxidize Fe(II). Msub_20060 encodes a putative ammonia monooxygenase, while Msub_12072 may encode a hydroxylamine

oxidoreductase in the *M. subterrani* genome. Ammonia is abundant and necessary in *M. subterrani* defined medium, and may act as an alternative electron source as well as producing reactive nitrogen species capable of Fe(II) oxidation. Additionally, *M. subterrani* encodes putative nitrate (*nar*, Msub_2280-2282) and nitrite (*nir*, Msub_3380-3383) reductases. These reductases produce reactive nitrogen species, and have been implicated directly with extracellular electron transfer in the Fe(II) reducing complex of *Aeromonas hydrophila* (B. Conley, personal communication, September 21, 2016).

Superoxide is unique among the reactive oxygen species. Despite being a powerful oxidant, superoxide can remain stable in circumneutral solution on the scale of minutes, allowing diffusion from cell surfaces before ultimately reacting with Fe(II) (Rose, 2012). Reacting at a distance from the cell surface maintains cell viability by avoiding entombment, thus the cell could maintain substantial Fe(II) oxidation, additionally benefiting from the localized decease in potentially toxic Fe(II) concentrations. While superoxide is a normal byproduct of terminal oxidase activity, respiratory reduction of molecular oxygen generates superoxide within the cytoplasm. As a charged molecule, the nominal quantity of superoxide generated by this mechanism is unable to diffuse through the inner membrane, and is rapidly scavenged by cellular defenses. However, several members of the genus *Marinobacter* produce substantial extracellular superoxide by a yet undetermined mechanism (Diaz et al., 2013). Extracellular superoxide generation is a trait shared by phylogenetically diverse bacteria, having implications towards element cycling beyond that of the influence of strict lithotrophs.

Materials and Methods

Media and culture conditions

When cultivated in rich medium, *Marinobacter subterrani* was grown at 30°C in Difco MB (Marine Broth) medium or LBMB (Luria-Bertani Marine Broth) medium contained 750 mL prepared Difco LB and 250 mL prepared Difco MB per liter. Defined-media conditions utilized *Marinobacter* Iron Medium (MIM) containing per liter: 50.0 g NaCl, 5.3 g MgCl2·6H2O, 0.75g KCl, 0.1 g MgSO4·6H2O, 50 mg K2HPO4, 1.0 g NH4Cl, 0.740 g CaCl2·2H2O, 0.42 g NaHCO3, 2.38 g HEPES (4-(hydroxyethyl)-1-piperazineethanesulfonic acid) and pH adjusted to 7.0 with 1 M HCl (Bonis and Gralnick, 2015). MIM was amended with 10 mM sodium lactate as the electron donor during heterotrophic cultivation in defined medium. *Escherichia coli* cultures were grown at 37°C in Difco LB (Luria-Bertani) medium. Media contained 50 μg/mL kanamycin sulfate (Fisher Scientific, MA, USA) for growth under kanamycin selection. Diaminopimelic acid was used at a concentration of 360 μM when growing the Diaminopimelic acid auxotrophic *E. coli* strain WM3064. Media were solidified with 1.5% (w/v) agar for growth on culture plates.

Growth conditions were assayed aerobically in MIM at 30°C unless varied for the conditions of the assay as noted. Heterotrophic growth of isolate strain JG233 in liquid media was monitored using optical density at 600 nm, unless the opacity of the media proved prohibitive, in which case growth was determined by enumeration of colony forming units (CFU) on LBMB solid medium. Medium for anaerobic assessment was prepared by sparging MIM with N_2 gas for 15 min in Balch tubes prior to autoclave sterilization.

Bacterial strains and mutant construction

The bacterial strains, plasmids, and oligomers used in this study are listed in Table 3.1. DNA modification enzymes were purchased from New England BioLabs, and oligomers were purchased from Integrated DNA Technologies. New England BioLabs Monarch DNA purification kits were utilized for all DNA purification steps. Mutant construction was performed using the protocol from Bonis and Gralnick, 2015 (Bonis and Gralnick, 2015). DNA flanking the regions of interest were amplified by polymerase chain reaction (PCR) using GoTaq Green Master Mix (Promega, WI, USA) in two reactions, one using primers UF/UR, and the other with DF/DR corresponding to the region of interest. Purified upstream and downstream amplicons were digested with the corresponding restriction endonucleases and ligated using T4 ligase into a similarly digested and purified pSMV3 vector (Saltikov and Newman, 2003) immediately prior to transformation into *E. coli* strain UQ950.

Purified deletion vector from *Escherichia coli* UQ950 was then used to transform donor strain *Escherichia coli* WM3064, which was then used to conjugate plasmids into *Marinobacter subterrani* recipients. Overnight cultures of donor and recipient were diluted to an optical density at 600 nm of 1.0 with LBMB. Donor cultures were washed once with LBMB to remove kanamycin, pelleted, and decanted. After a 45° C heat shock for 5 min, 1 mL recipient culture was used to resuspend donor pellets. Cultures were again pelleted, resuspended in 100 μL LBMB, and spotted to LBMB plates. Following an 18 h incubation at 30° C, spots were resuspended in LBMB and spread to MB plates

devoid of diaminopimelic acid and amended with kanamycin for selection against the donor strain and for the selection of merodiploid mutants, respectively. Kanamycin

Table 3.1. Bacterial strains, vectors, and oligomers		
	Relevant Traits	Source
Marinobacter subterrani		
JG233	Wild Type	This Study
JG2758	ΔMsub_10588-10600	This Study
JG2759	ΔMsub_12037-12038	This Study
JG3129	JG2759 pBBR1MCS2::Msub_12037-12038	This Study
JG3420	ΔMsub_12072	This Study
JG3485	ΔMsub_12632	This Study
JG3419	ΔMsub_20060	This Study
JG3468	ΔMsub_2280-2282	This Study
JG3452	ΔMsub_3380-3383	This Study
Escherichia coli		
UQ950	DH5α vector construction host	1
WM3064	DAP auxotroph donor strain for conjugation	1
Vectors		
pSMV3	Deletion vector, Km^r, *sacB*	1
pBBR1MCS-2	Broad range cloning vector, Km^r	2
pSMV3ΔMsub_10588-10600	ΔMsub_10588-10600 deletion construct	This Study
pSMV3ΔMsub_12037-12038	ΔMsub_12037-12038 deletion construct	This Study
pBBR1MCS2::Msub_12037-12038	ΔMsub_12037-12038 complementation vector	This Study
pSMV3ΔMsub_12072	ΔMsub_12072 deletion construct	This Study
pSMV3ΔMsub_12632	ΔMsub_12632 deletion construct	This Study
pSMV3ΔMsub_20060	ΔMsub_20060 deletion construct	This Study
pSMV3ΔMsub_2280-2282	ΔMsub_2280-2282 deletion construct	This Study
pSMV3ΔMsub_3380-3383	ΔMsub_3380-3383 deletion construct	This Study
Oligomers		
ΔMsub_10588-10600 UF	CATG**GGATCC**CTTGGCTTTGTCGAATGGGTGG	This Study
ΔMsub_10588-10600 UR	CATG**CTCGAG**GGTTAGACTAATCTCAGCGG	This Study
ΔMsub_10588-10600 DF	CATG**CTCGAG**TTCTGATTTTCAATCGAACC	This Study
ΔMsub_10588-10600 DR	CATG**ACTAGT**GTTACCAGCTTTATTCAACCC	This Study
ΔMsub_12037-12038 UF	CGAT**GAGCTC**ACCTATGTCCTGGTCAACATCG	This Study
ΔMsub_12037-12038 UR	CGAT**CCATGG**TGTCATTGTTCGCCTCCAATA	This Study
ΔMsub_12037-12038 DF	CGAT**CCATGG**GAATAAGGTCACAACGGGCAG	This Study
ΔMsub_12037-12038 DR	CGAT**ACTAGT**TTGTTGGGCATCTACTATCC	This Study
::Msub_12037-12038 UF	CGAT**GAGCTC**ACCTATGTCCTGGTCAACATCG	This Study
::Msub_12037-12038 DR	CGAT**ACTAGT**CTGCCCGTTGTGACCTTATTC	This Study
ΔMsub_12072 UF	CGAT**GGATCC**CGGTGCCGAAGTGCAGGTCAGGC	This Study
ΔMsub_12072 UR	GCAT**CTTAAG**TGTCATTTGCGTTCTCCACTGTTGC	This Study
ΔMsub_12072 DF	CGAT**CTTAAG**TCCTGATACAGCCTGGGCCCCACC	This Study
ΔMsub_12072 DR	CGAT**GAGCTC**TCACGGTTTTTGACGGCGTTTGGC	This Study
ΔMsub_12632 UF	CGAT**ACTAGT**CTTGCTCACCCAGAACAACGGC	This Study
ΔMsub_12632 UR	CGAT**CTTAAG**CATCTGTGTTACTCCAAACTACACG	This Study
ΔMsub_12632 DF	CGAT**CTTAAG**GAGGTATGAGCTGTATTCG	This Study
ΔMsub_12632 DR	CGAT**GAGCTC**CCTGCAATGTGCCATTTGGC	This Study
ΔMsub_20060 UF	ACGT**GGATCC**GGAGGTTCTCCACTGTAAGTGGG	This Study
ΔMsub_20060 UR	ACGT**CTTAAG**AATCACGTCAGTCTAATCATGG	This Study
ΔMsub_20060 DF	ACGT**CTTAAG**AATTGAGGTAGTGCCGTTGCCC	This Study

ΔMsub_20060 DR	ACGT**GAGCTC**GCAACTACATCTGCTCAGCGG	This Study
ΔMsub_2280-2282 UF	GCTA**GGATCC**CTCGGTCAGGGTCCTGCTGCG	This Study
ΔMsub_2280-2282 UR	CGTA**CTTAAG**ACTCATGGTCTCTCTCTCCG	This Study
ΔMsub_2280-2282 DF	CGAT**CTTAAG**GCGTAATGGTGTGAATTGAGGC	This Study
ΔMsub_2280-2282 DR	CGAT**GAGCTC**CGGCTCTCGATCAGTTCCG	This Study
ΔMsub_3380-3383 UF	CGAT**GGATCC**TTGTCCGGCTGGCGACACAGC	This Study
ΔMsub_3380-3383 UR	CGAT**CTTAAG**CTTCATCGTGTTCAATCCCG	This Study
ΔMsub_3380-3383 DF	CGAT**CTTAAG**GCATAATCTGAACTTACCG	This Study
ΔMsub_3380-3383 DR	CGAT**GAGCTC**CAGGGCATCCTGATTCATCAGG	This Study
M13F	GTA AAA CGA CGG CCA GT	3
M13R	CAG GAA ACA GCT ATG AC	3

1. Saltikov and Newman (2003)
2. Kovach et al. (1995)
3. "Universal Primer List" (n.d.)

resistant colonies were verified by PCR using insertion-specific primers. Selection on LBMB plates amended with 400 mM sucrose yielded kanamycin-sensitive colonies. Deletion mutants were confirmed by Sanger sequencing across the deleted region.

Iron quantification

Fe(II) concentration was determined colorimetrically using ferrozine reagent and quantified spectrophotometrically at 562 nm (Stookey, 1970). Fe(II) was stabilized against abiotic oxidation by mixing 300 µL of sample with 50 µL of 5 M HCl in a 96-well, clear-bottomed plate. Stabilized samples were diluted to appropriate concentrations in 0.5 M HCl before 50 µL aliquots were thoroughly mixed with 300 µL of prepared ferrozine reagent containing 2 g 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid monosodium salt hydrate (Sigma-Aldrich, MO, USA) and 23.8 g HEPES per liter immediately prior to spectrophotometric reading.

Cultivation with Fe(II)

The gel-stabilized gradient tube system from Emerson and Floyd (2005) was adapted for Fe(II) oxidation assays (Emerson and Floyd, 2005). In brief, FeS stabilized in

1.0% agarose was deposited in the bottom of sterile Balch tubes and allowed to solidify. Alternative Fe(II) sources included Fe^0, $FeCl_2$, $Fe(CO_3)_2$, and $FeSO_4$. A low-melt agarose (SeaKem LE Agarose, Lonza, ME, USA) stabilized MIM at pH 7.0 and lacking HEPES, was gently overlaid on the Fe(II)-plugs and gassed for 1 min using 20% CO_2 gas with a balance of N_2. Tubes were then stoppered with butyl rubber bungs and allowed to set overnight at ambient temperature. Heterotrophically grown cultures were washed in defined medium lacking organic carbon before inoculation into the gradient tubes. Inoculation was performed by removing the bung from the tube, allowing atmospheric gas into the headspace, and injecting cells throughout the length of the tube. Gradient tubes were incubated statically in the dark at 30° C.

To determine colony forming unit density in gradient tubes, the entire content of the gradient tube was homogenized by passage through a 25-gauge needle immediately prior to plating on LBMB. Colony forming unit distribution was accomplished by sectioning gradient tubes into 1 cm segments (approximately 1.5 mL). Sections were individually homogenized prior to plating on LBMB.

Chemolithoorganotrophic cultivation with Fe(II)

Cultivation in liquid culture was done using 18 mm Balch tubes containing 15 mL MIM medium and 10 mM sodium acetate and/or 10 mM FeS added after autoclaving. The headspace was sparged with 1% (v/v) dioxygen with a nitrogen balance. Cell enumeration was accomplished by removing 100 µL aliquots and plating for colony forming units on LBMB plates and minimum probable number dilution series in LBMB.

Gradient tubes were amended with 1, 10, 100, or 1000 µM sodium acetate to assess for chemolithoorganotrophic utilization of Fe(II) in established Fe(II)-oxidizing conditions. Tubes were set up in series and individual tubes sacrificed to determine total colony forming units.

Nitrate quantification

Using a previously described method (Ringuet et al., 2011), 100 µL aliquots of culture were deposited in 96-well, clear-bottomed plates. Following the addition of 50 µL each of 58 mM sulfanilamide (Sigma-Aldrich, MO, USA) in 3.6N hydrochloric acid and 3.86 mM N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich, MO, USA) samples were incubated at ambient temperature for 15 minutes following thorough mixing. Absorbance readings were taken using a Molecular Devices SpectraMax M5 spectrophotometer at 540 nm against a sodium nitrite standard.

Extracellular superoxide activity

Detection of superoxide used a method adapted from Godrant et al, 2009 (Godrant et al, 2009). 10 µL of ambient temperature 3 mM stock solution of diethylenetriaminepentaacetic acid (DTPA) was added to the bottom of a sterile 1.5 mL microcentrifuge tube. In the blank, 5 µL of a 3 kU/mL superoxide dismutase (Fisher Scientific, NH, USA) stock was added. Standards were amended to 1.5mU/L, 8mU/L, and 20mU/L final concentration with freshly prepared 3U/L xanthine oxidase (Roche Diagnostics, IN, USA) solution in phosphate buffer saline, but with the drop suspended on the wall of the microcentrifuge tube, as to prevent mixing with the DTPA. Just prior to

total luminescence detection in a Promega GloMax 20/20 luminometer, 280 µL of culture in defined medium was added, allowing the contents to mix thoroughly. Following the addition of 12 µL of 250 µM [2-[4-[4-[3,7-dihydro-2-methyl-3-oxoimi-dazo[1,2-a]pyrazin-6-yl]phenoxy]butyramido]ethylamino]sulforhodamine101 (red-CLA; Tokyo Chemical Industry, Tokyo, Japan) in 50 % (v/v) ethanol in water, the sample was assayed for total luminescence over 1 minute, with a 1 second data acquisition time.

Assessment of the effect of superoxide on Fe(II) oxidation was done in gradient tube systems. Cultures were prepared as described previously (Bonis and Gralnick, 2015) and amended with superoxide dismutase or xanthine oxidase to a final activity of 532.5 U/L and 20 mU/L, respectively. Tubes with xanthine oxidase additionally contained xanthine to a final concentration of 50 µM.

Adenosine triphosphate analysis

For liquid media, a 250 µL aliquot of culture was pelleted and resuspend in 100 µL of 1% trichloroacetic acid followed by a 2 hour incubation on ice. Aliquots from gel solidified cultures were first centrifuged at 10,000 xg for 5 minutes, the liquid supernatent removed, and the gel pellet resuspended in 400 µL 1% trichloroacetic acid before the 2 hour incubation on ice. 10 µL of extracted sample, 100 µL of 15 mM Tris-acetate pH 7.75, and 100 µL of rL/L reagent from the Enliten ATP Concentration kit (Promega, WI, USA) were combined immediately prior to total luminescence detection in a Promega GloMax 20/20 luminometer with a 10 second interval with integrated reading every second. Aliquots were removed from original cultures and plated to determine colony forming units as well.

Results and Discussion

Candidate genes for metabolic Fe(II) oxidation

Marinobacter subterrani mutants Msub_12037-12038, Msub_12632, Msub_10588-10600, Msub_20060, Msub_12072, Msub_3380-3383, and Msub_2280-2282 displayed wild type Fe(II) oxidation in gradient tubes, suggesting these genes do not encode products essential to Fe(II) oxidation in *M. subterrani*. The possibility remains that Fe(II) oxidation in *M. subterrani* is multivariate, and the disruption of a single contributing system is insufficient to measurably alter activity, or a certain degree of compensatory plasticity masks the effects. However, the mechanism of Fe(II) oxidation in *M. subterrani* is likely mediated by a mechanism unlike those suggested in the literature.

No growth benefit is observed when Marinobacter subterrani *is cultivated on Fe(II)*

Marinobacter subterrani lacks a described mechanism for autotrophic growth encoded in the genome, suggesting any electrogenic benefits of Fe(II) oxidation may be observed instead during chemolithoorganotrophic cultivation. Electrogenic benefit would manifest in an increased growth rate or higher overall cell density, as ATP or reducing equivalents produced through Fe(II) oxidation would supplement anabolic metabolism provided from acetate oxidation alone. An increase in growth rate or final cell density is not observed in either liquid or gradient tube cultivation with acetate and Fe(II) (Figure 3.2), suggesting Fe(II) oxidation does not contribute an anabolic benefit. A lack of chemolithoorganotrophy on Fe(II) is supported by cellular concentrations of ATP in the presence of Fe(II) (Figure 3.3). The decreased cellular ATP concentrations suggest not

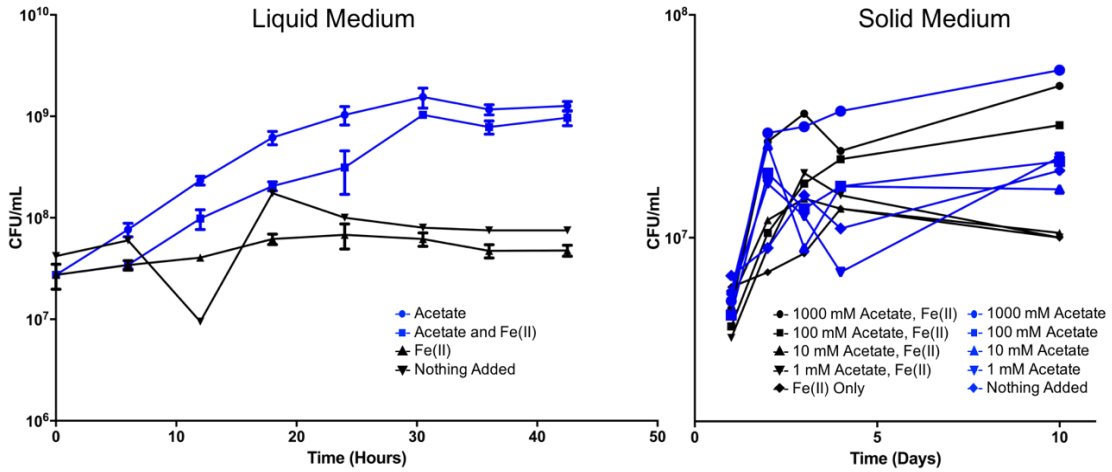


Figure 3.2. Assessment for Fe(II) chemolithoorganotrophy in *Marinobacter subterrani.* Cultivation in liquid and gradient tube (solid) culture in 18 mm Balch tubes containing 15 mL MIM medium. Liquid culture utilized a 1% (v/v) dioxygen with a nitrogen balance headspace with 10 mM sodium acetate and 10 mM FeS. Cell enumeration was accomplished by removing 100 µL aliquots and plating for colony forming units on LBMB plates and minimum probable number dilution series in LBMB. Solid medium utilized gradient tubes amended with 1, 10, 100, or 1000 µM sodium acetate. Individual tubes from each series were sacrificed for cell enumeration by colony forming units.

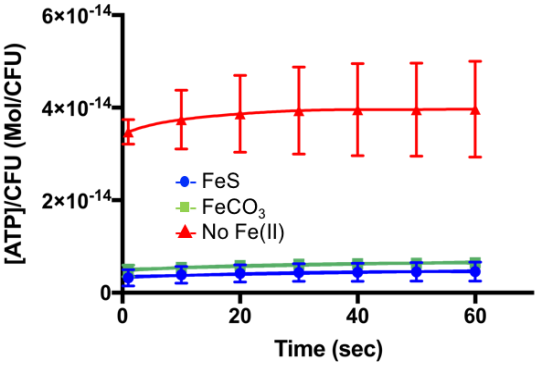


Figure 3.3. Effect of Fe(II) on cellular adenosine triphosphate concentrations. Adenosine triphosphate was extracted using trichloroacetic acid from gradient tube incubated cells. Extracted samples were assayed using the Enliten ATP Concentration kit (Promega, WI, USA) on a Promega GloMax 20/20 luminometer with a 60 second interval with integrated reading every second. Error bars are shown only for 10 second intervals.

only does *M. subterrani* not gain an electrogenic advantage from Fe(II) oxidation, but may actually cause an additional burden to the cell.

In the absence of an electrogenic advantage of Fe(II) oxidation in *M. subterrrani*, the mechanism of Fe(II) oxidation may be inferred by the relationship between iron and potentially involved reactants. Determining the cell concentration capable of producing reliable Fe(II) oxidation becomes important to distinguish the effects of possible cell produced reactants. In experiments attempting to determine minimum effective titer, growth of *M. subterrani* in the absence of additional carbon source was still observed.

Cells incubated in defined medium without added carbon source until cellular concentrations of ATP leveled out recovered and grow upon transfer to new defined medium without added carbon source. Attempts to minimize organic carbon contamination through the use of new, acid and based-washed, muffle-furnace cleaned glassware, HLPC grade water, and new reagents were unsuccessful. *M. subterrani* has also been demonstrated of being incapable of using HEPES as an alternative electron, carbon, nitrogen, or sulfur source.

Effect of byproducts on Fe(II) oxidation

In the millimolar quantities capable of supporting substantial growth, the respiration of nitrate to nitrite has been suggested as a possible mechanism of microbial-mediated Fe(II) oxidation (Carlson et al., 2013; Klueglein and Kappler, 2012). While Fe(II)-oxidation in *M. subterrani* is oxygen-dependent, MIM-grown cultures generate micromolar (1-10 µM) quantities of nitrite through an unknown mechanism. However, additional nitrate or nitrite up to 10 µM failed to replicate *M. subterrani*-like Fe(II) oxidation in gradient tubes, and indeed showed no effect up to 100 µM (Figure 3.4). Deletion mutants of genes encoding proteins potentially involved in generation of reactive nitrogen species, Msub_20060, Msub_12072, Msub_3380-3383, and Msub_2280-2282, all generated similar levels of nitrite as wild type *M. subterrani* and displayed no loss in Fe(II) oxidation capacity.

Deletion of Msub_10588-Msub_10600, the putative *sox* operon, had no effect on Fe(II) oxidation in *M. subterrani*. This is supported by the capacity of *M. subterrani* to

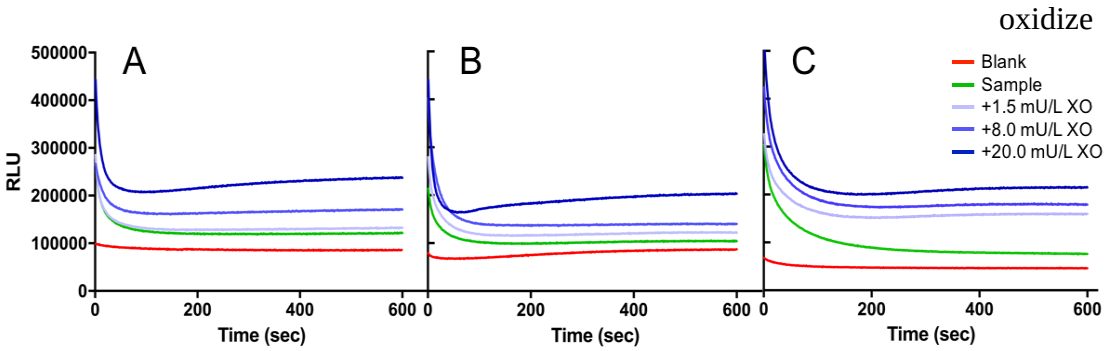


Figure 3.4. Representative superoxide production by *M. subterrani*. Superoxide production was quantified by luminescence from the reaction of superoxide with the dye [2-[4-[4-[3,7-dihydro-2-methyl-3-oxoimi-dazo[1,2-a]pyrazin-6-yl]phenoxy]butyramido]ethylamino]sulforhodamine101. Samples are compared with internal standards of xanthine oxidase produced superoxide. **A**. Representative superoxide production by wild type *M. subterrani*. **B**. Representative superoxide production by *M. subterrani* ΔMsub_12037-12038. **C**. Representative superoxide production by *M. subterrani* ΔMsub_12632. Abbreviations: RLU, Relative Fluorescence Units; XO, Xanthine Oxidase.

Fe(II) sources containing no sulfur species, including $FeCl_2$, $FeCO_3$, and zero valent iron.

Superoxide has been implicated as an environmentally relevant manganese oxidant, and may react similarly with Fe(II) (Learman et al., 2011; Nealson, 2006). Like marine species of the same genus, *M. subterrani* produces extracellular superoxide (Figure. 3.4a) through an unknown mechanism. Deletion mutants of genes encoding potential superoxide generating enzymes, Msub_12037-12038 and Msub_12632, were able to generate similar levels of superoxide as wild type *M. subterrani* (Figure 3.4b and 3.4c, respectively), and displayed similar Fe(II) oxidation, and thus are unlikely to be involved in either process. Alternatively, *in situ* generation of additional superoxide using xanthine oxidase did not observably accelerate Fe(II) oxidation in a gradient tube system. While this does not conclusively demonstrate *M. subterrani* does not mediate Fe(II) oxidation via extracellular superoxide generation, it does establish that under conditions

were *M. subterrani* mediates Fe(II) oxidation, either superoxide is unable to oxidize Fe(II) or xanthine oxidase is unable to generate superoxide.

Though the specific components facilitating Fe(II) oxidation in *M. subterrani* remain elusive, the lack of any observable metabolic benefit from Fe(II) oxidation suggests it proceeds through an active mechanism. Much of focus on dissimilatory Fe(II) oxidation has focused on metabolic mechanisms under the assumption lithoautotrophic organisms mediate the majority of biotic Fe(II) oxidation. The ubiquity and abundance of the *Marinobacter* in diverse environments suggests a better understanding of iron cycling must encompass a broader view of contributing factors.

Chapter 4: Expanding the Utility of High Throughput Transposon Mutagenesis in *Marinobacter*

Summary

High throughput sequencing of transposon mutant libraries enables the cataloging of the conditional effect of each genetic element on the growth or survival of an organism. Here we establish the use of homopolymer tailed transposon sequencing to ascribe genetic contribution to growth in *Marinobacter subterrani* under rich and defined media growth conditions. DEATH-seq, a growth-independent adaptation of Tn-seq is developed and implemented to determine *M. subterrani* genetic elements contributing to survival in non-growth conditions. Finally, concepts from *M. subterrani* are applied to five additional members of the genus *Marinobacter* to produce transposon mutant libraries. Genes essential for growth are derived by sequencing these transposon mutant libraries, and then compared to determine essential pan- and core-genomes.

Introduction

Mutagenesis techniques

Targeted mutagenesis techniques are vital for verifying phenotypic outcomes of genetic elements, however the considerable resource expenditure involved in directed mutagenesis prohibits use at scale in most organisms and requires knowledge of the genetic components involved. Using nonspecific approaches such as chemical, physical, or mobile element mutagenesis allows for rapid genome-wide analysis, requires no *a priori* knowledge of genetic components, and is largely researcher-unbiased. As different mutagens induce mutation through specific chemistry, interaction, or mechanism, careful selection of mutagen has a large effect on outcome. Chemical and physical mutagens tend to react with specific nucleotides independent of surrounding sequence, often resulting in

single base or dinucleotide changes at loci (Lawrence, 2002). Mutations arising from chemical and physical mutagenesis are difficult to identify and interpret, as this technique often makes a plethora of small dispersed genetic changes. However, these small mutations better mimic the natural course of mutational evolution, and result in a higher frequency of gain of function, less severe loss of function, and frameshift mutations (Lawrence, 2002). Transposable elements insert semi-randomly, but leave easily identified and customizable features for analysis. Sizable enough to disrupt normal function upon insertion into a genetic element; transposons rarely result in functional products with altered activity, limiting screening to loss of function analysis. However, transposons can encode features such as additional mobile elements, antibiotic resistance and other selectable markers, and regulatory elements. Careful selection of the transposon employed can also regulate the number of disruptions per organism, specificity of insertion site, inverted repeat sequence, cross-reactivity, and host range.

The pMiniHimar RB1 transposon cassette

A *mariner*-type transposon, MiniHimar transposes by a type-II mechanism of excision and insertion (Muñoz-López and García-Pérez, 2010), duplicating almost exclusively during host replication or homologous recombination during transposition. Type-II transposons lend well to transposon mutagenesis screens as typically only a single disruption event is desired per organism. Additionally, *mariner* subfamily transposases catalyze both excision and insertion, allowing a broad host range and functioning in a variety of eukaryotic and prokaryotic hosts (Muñoz-López and García-Pérez, 2010; Bouhenni et al., 2005, Lampe et al., 1998). Coupled to broad host range,

Himar transposons are well suited for mutagenesis studies as dinucleotide TA sites are the favored insertion sequence, and are broadly distributed throughout individual genomes and across species. The pMiniHimar RB1 vector (Figure 4.1) is designed with a neomycin phosphotransferase cassette and an R6Kγ origin of replication targeted for insertion into the genome via inverted repeats (Bouhenni et al., 2005). The R6Kγ origin limits replication of the plasmid to organisms carrying the *pir* genes, restricting copy number in host organisms, and thus limiting mutations to a single event per organism (Rubin et al., 1999). The neomycin phosphotransferase acts as a selectable marker for cassette insertion. External to the inverted repeats, and thus not targeted for transposition, are an origin of transfer and a gene encoding the transposase (Bouhenni et al., 2005). This positioning allows for conjugative transfer of the vector along with all the machinery required for transposition, while assuring these portions will not be replicated via insertion into the host genome.

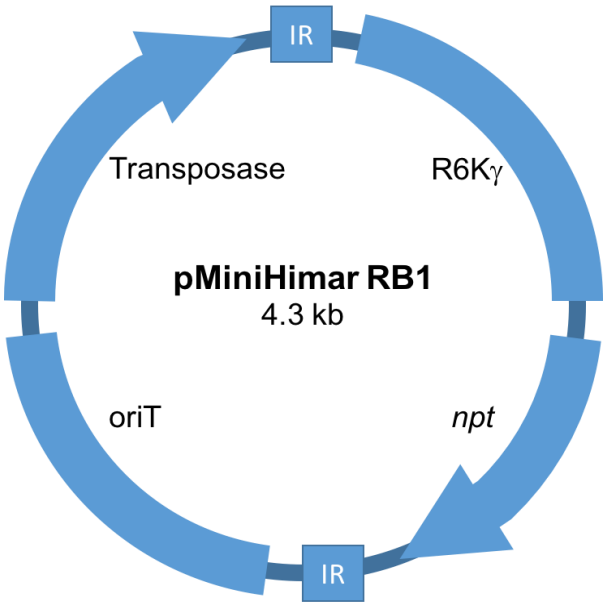


Figure 4.1. Plasmid map of vector pMiniHimar RB1. The R6Kγ origin of replication and kanamycin resistance encoding *npt* are targeted for transposition by the inverted repeats. The transposase and origin of transfer remain, and are therefore left unintegrated. Once transposition occurs, excising the R6Kγ origin of replication, the vector is no longer able to replicate. Abbreviations: IR, inverted repeat; oriT, origin of transfer; *npt*, neomycin phosphotransferase.

Tn-seq: Transposon sequencing

Knowledge of conditional essentiality and genetic contribution to phenotype are central to genetic engineering and developing a framework for understanding metabolic interactions. As individual metabolic reactions and pathways have a global effect on regulation and flux; an encompassing knowledge of the contribution of each genetic element in an organism under a condition grants insight into the mechanisms utilized by the organism under that condition, as well as suggesting phenotypic roles for genetic elements. Transposon sequencing, or Tn-seq, identifies the location of individual transposon insertions on a population scale using high-throughput sequencing. By determining how selective conditions effect relative abundance of mutant populations, inferences can be made regarding the contribution of each disrupted genetic element to the overall ability of the organism to replicate under that condition. Though several iterations are now in practice, the concept developed by Opijnen et al., 2009 remains a cohesive theme (Opijnen et al., 2009, Kwon et al., 2015). A saturated mutant library is constructed and is grown under a condition of interest. DNA isolated from the parental and outgrown populations is selectively amplified; enriching a population of amplicons containing using transposon-specific oligomers and polymerase chain reaction. The amplicons are sequenced, genomic DNA immediately adjacent to the transposon is mapped to the genome, and the reads corresponding to individual insertion locations are tallied. Assuming transposon insertion is sufficient to abolish function of the disrupted genetic element, the difference in relative abundance corresponding to each genomic location can be used to infer the contribution of that location to relative growth rate of the organism. To establish the viability of the technique in *Marinobacter subterrani*,

selective conditions of growth in rich and defined medium conditions were used for outgrowth

DEATH-seq: Determining endurance attributes by transposon high-throughput sequencing

Growth and death rates modulate population size, thus enduring genetic factors contribute to maintaining viability as well as growth. In complement to the growth-selective assay of Tn-seq, DEATH-seq determines genes contributing to robustness under specific selecting conditions. A saturated transposon mutant library is subjected to a selective condition facilitating the loss of cell viability by several orders of magnitude. Genomic DNA adjacent to transposon sequence is sequenced in parental and surviving populations, and the relative abundance between populations can be used to infer the contribution of genetic elements to enduring the selective condition. As the final population is considerably decreased, a subsequent outgrowth may be required to obtain sufficient genomic DNA for analysis.

Comparative essential gene profiling

The gradual accumulation of mutations that is the foundation of evolution suggests the genetic content of members of a species will drift under defining selective pressures while maintaining similar features. Genes essential under a condition are maintained, but once selective pressure is removed, these same genes may progress to non-essential, change activity, or alter regulation. Genomic comparison is a powerful tool for the study of evolution, but fails to differentiate between genomic content and utility to

the organism. Members of the genus *Marinobacter* are found in a variety of highly selective environments regarding salinity, temperature, carbon sources, and electron sources. Thus, orthologues may be present, but maintain different importance to the survival of respective hosts. Here we propose the use of comparative essential gene profiling to provide a more diagnostic approach to the full genome comparison of organisms. Transposon insertion libraries are prepared in several *Marinobacter* to determine and compare the utility of genomic content.

Materials and Methods

Media and culture conditions

When cultivated in rich medium, *Marinobacter* strains were grown at 30°C in LBMB (Luria-Bertani Marine Broth) medium contained 750 mL prepared Difco LB and 250 mL prepared Difco MB per liter (Bonis and Gralnick, 2015). Defined-media conditions utilized *Marinobacter* Iron Medium (MIM) containing per liter: 50.0 g NaCl, 5.3 g MgCl2·6H2O, 0.75g KCl, 0.1 g MgSO4·6H2O, 50 mg K2HPO4, 1.0 g NH4Cl, 0.740 g CaCl2·2H2O, 0.42 g NaHCO3, 2.38 g HEPES (4-(hydroxyethyl)-1-piperazineethanesulfonic acid) and pH adjusted to 7.0 with 1 M HCl (Bonis and Gralnick, 2015). MIM was amended with 10 mM sodium lactate as the electron donor during heterotrophic cultivation in defined medium. *Escherichia coli* cultures were grown at 37°C in Difco LB (Luria-Bertani) medium. Media contained 50 μg/mL kanamycin sulfate (Fisher Scientific, MA, USA) for growth under kanamycin selection. Diaminopimelic acid was used at a concentration of 360 μM when growing the diaminopimelic acid

auxotrophic *E. coli* strain WM3064. Media were solidified with 1.5% (w/v) agar for growth on culture plates.

A single aliquot of the library stock was recovered in 10 mL LBMB for 15 minutes at 30 ^{o}C for Tn-seq analysis in *Marinobacter subterrani*. Culture was then washed thrice in either MIM or LBMB before inoculating MIM or LBMB, respectively, to an optical density at 600 nm of 0.01. The remaining starter culture was frozen immediately at -80 ^{o}C to serve as the parental population. Outgrowth conditions were harvested at 5 doublings and frozen immediately at -80 ^{o}C until DNA extraction

Cultures during *M. subterrani* DEATH-seq analysis were incubated in unsterilized Soudan Iron Mine water from Diamond Drill Hole 942. Water was collected and maintained anaerobic at 4 ^{o}C until use. Library stocks were recovered in LBMB prior to washing thrice with unsterilized mine water to a final titer of 2.5 x 10^{7} CFU/mL. Cultures were incubated 72 hours at 30 ^{o}C without shaking. Final cell culture was isolated by centrifugation and half frozen immediately at -80 ^{o}C. The remaining culture was resuspended in 50 mL of oxic LBMB for two hours at 30 ^{o}C prior to storage at -80 ^{o}C.

Transposon Library Construction

Bacterial strains, vectors, and primers used in this study are listed in table 4.1. Optical density measurements were taken at 600nm. Conjugation of pMiniHimar RB-1 into *Marinobacter* strains was performed as described in Bonis, 2015. Briefly; recipient *Marinobacter* cultures of optical density 1.0 were incubated at 45^{o}C for 5 minutes before mixing with an equal number of Escherichia coli WM3064 carrying the pMiniHimar RB-

1 vector. Cells were then centrifuged and resuspended 10X, and spotted to LBMB plates with 360 μM diaminopimelic acid. Mating spots were resuspended in LBMB following an 18 hour incubation and spread to LBMB. Resulting colonies were resuspended in LBMB with 15% glycerol and stored at -80^{o}C until use. Libraries from *Marinobacter hydrocarbonoclasticus*, *Marinobacter* sp. CP1, and *Marinobacter persicus* were outgrown an additional two doublings in rich medium to minimize *E. coli* strain WM3064 gDNA contamination.

DNA Processing

DNA preparation for sequencing is summarized in Figure 4.2 and is adapted from that described in Klein et al., 2012 (Klein et al., 2012). Cells were harvested by centrifugation and DNA purified by phenol-chloroform extraction. Subsequent DNA

Table 4.1. Bacterial strains, vectors, and oligomers

	Relevant Traits	Source
Strains		
Escherichia coli	Strain WM3064; DAP auxotroph donor strain for conjugation	1
Marinobacter subterrani	Strain JG233	2
***Marinobacter* sp. CP-1**		3
Marinobacter hydrocarbonoclasticus	Strain ATCC 49840	4
Marinobacter persicus	Strain UTICA-S1B3	5
***Marinobacter* sp. JG228**		This study
***Marinobacter* sp. FeSN6p**		6
Vector		
pMiniHimar RB-1	Vector bearing a transposon borne kanamycin resistance cassette	7
Oligomers		
Rnd1_TnF	CGAGTTCTTCTGAGCGGGACTC	This Study
Rnd1_AdaptR	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGGGGGGGGGGGGGGG	This Study
Rnd2F	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGACCGGGGACTTATCAGCCAACCTG	This Study
Rnd2BCt1R	CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC	This Study

Rnd2BCt2R	CAAGCAGAAGACGGCATACGAGAT**GATCTG**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATC	This Study
Rnd2BCt3R	CAAGCAGAAGACGGCATACGAGAT**TTTCAC**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATC	This Study
Rnd2BCt4R	CAAGCAGAAGACGGCATACGAGAT**CTCTAA**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATC	This Study
Rnd2BCt5R	CAAGCAGAAGACGGCATACGAGAT**GAGCCC**GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATC	This Study
Rnd2BCt6R	CAAGCAGAAGACGGCATACGAGAT**CCGAGA**GTGACTGGAGTTCAGA CGTGTGCTCTTCCGATC	This Study
Barcode Assignment		
Rnd2BCt1R	*Marinobacter subterrani* Parental	
Rnd2BCt2R	LBMB, T1, and *Marinobacter* sp. FeSN6p	
Rnd2BCt3R	MIM, T2, and *Marinobacter hydrocarbonoclasticus*	
Rnd2BCt4R	*Marinobacter* sp. JG228	
Rnd2BCt5R	*Marinobacter persicus*	
Rnd2BCt6R	*Marinobacter* sp. CP-1	

1. Saltikov and Newman (2003)
2. Bonis and Gralnick (2015)
3. Wang et al. (2015)
4. Gauthier et al. (1992)
5. Paula Mouser, Ohio State
6. Rowe, et al. (2015)
7. Bouhenni et al. (2005)

purification steps were performed using the New England BioLabs Monarch Nucleic Acid Purification Kit Series. DNA was sent to the University of Minnesota Genomics Center for shearing to 1Kb using a Covaris S220 Focused-ultrasonicator. Polycytosine tails were added to sheared fragments using terminal transferase (NEB, MA), 1X TdT reaction buffer, 500 μM deoxycytidine triphosphate, and 50 μM dideoxycytidine triphosphate to restrict the tail length to approximately 20 bases. Fragments containing transposons were selectively amplified by polymerase chain reaction using GoTaq Green (Promega; WI, USA), Phusion Polymerase (NEB; MA, USA), and primers Rnd1_TnF and Rnd1_AdaptR (Table 4.1). Amplicons within the linear range of the polymerase chain reaction were purified and subsequently amplified using primers Rnd2_F and Rnd2_BCXR, where X is an indicator of the incorporated

barcode for multiplexing (Table 4.1). Amplicons isolated from the linear range of the amplification were purified and sequenced using an Illumina HiSeq 2500 125bp PE flow cell using v4 chemistry by the University of Minnesota Genomics Center.

Bioinformatic analysis

Bioinformatic processing and analysis was accomplished using the Galaxy platform (www.usegalaxy.org) supported by the Minnesota Supercomputing Institute at the University of Minnesota. Genomic DNA sequence 30 bp immediately adjacent to the transposon was mapped to the corresponding genome (table 4.2) using Bowtie (www.bowtie-bio.sourceforge.net) with default parameters allowing three mismatches in the seed. Loci with single insertions were removed from the results to minimize the inclusion of mapping errors.

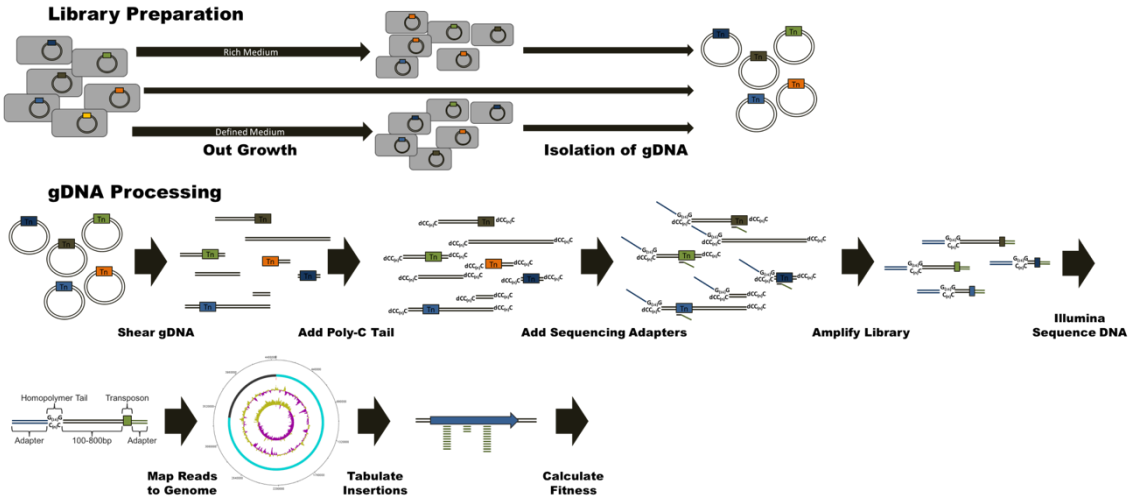


Figure 4.2. Workflow of homopolymer tailed Tn-seq. Parental transposon mutant libraries are grown under selecting conditions. Total genomic DNA is then isolated and shorn to 1kb. The resulting fragments are poly-cytosine tailed prior to selective amplification by polymerase chain reaction. Adapter DNA is added via the primers used for amplification. The resulting DNA is sequenced on a high throughput platform, mapped to the host genome, and reads are tallied per insertion site. Comparison of parental to conditional insertion ratios are used to calculate the effect of transposon insertion on growth.

Essential gene comparison was conducted using the GET_HOMOLOGUES software package (Contreras-Moreira and Vinuesa, 2013). Essential gene profiles were prepared as amino acid fasta files and the matrix prepared using the OrthoMCL clustering algorithm with a minimum full gene length identity of 30% and accounting for Pfam domains.

Results and Discussion

Library Preparation

Library preparation results are summarized in Table 4.2. Sanger sequencing of 16S rDNA and transposon adjacent genomic DNA yielded reads that mapped back to the corresponding genome; confirming both species identification as well as genomic integration of the transposon.

Table 4.2 Library parameters for *Marinobacter* strains.

	1	2	3	4	5	6
Mutant Diversity (CFU)	1.5×10^5	2.3×10^5	2.8×10^5	4.5×10^4	1.8×10^5	2.1×10^5
Cell Density (CFU/mL)	2.0×10^{11}	5.5×10^9	3.0×10^{11}	3.0×10^{10}	2.1×10^{10}	3.0×10^{11}

1. *Marinobacter subterrani JG233*
2. *Marinobacter* sp. CP1
3. *Marinobacter* sp. JG228
4. *Marinobacter*sp. FeSN6p
5. *Marinobacter persicus* UTICA-S1B3
6. *Marinobacter hydrocarbonoclasticus* ATCC 49840

Tn-seq

Reads from the parental library mapped to 3,839 of the 4,222 putative genes encoded in the *M. subterrani* genome with a TA site saturation of 62.8%. As library construction involves growth in LBMB, it is likely the majority of the 383 genes not represented in the parental population are essential under the rich medium conditions

used during library preparation. These findings are consistent with essential gene estimates in other organisms; between 346 and 398 essential genes in *Streptococcus pneumoniae* (Opijnen et al., 2009), 358 in *Haemophilus influenzae* (Gawronski et al, 2009), 382 in *Mycoplasma genitalium* (Glass et al., 2006), and 358 genes in *Escherichia coli* K-12 (Goodall at al., 2018). Corroborating these results, no additional genes were found to be essential for growth on LBMB in the rich medium outgrowth, though gene coverage was reduced to 2,817 genes. Though it is possible additional conditionally essential genes are present in this unrepresented population, it is more likely transposon disruption in these genes resulted in the more severe deleterious growth effects. As the parent population had been selected on rich medium, the genes contributing the most to growth would have been diminished in the starting population, and further reduced during additional selection on rich medium.

Generally, the flexibility afforded when growing in rich medium is considered a benefit to cells, however, selection in minimal medium had a greater number of gene disruptions benefitting growth than the rich medium condition. Of the 2,314 genes containing significant mapping data, 1,604 genes apparently improved growth rate when disrupted, as compared to only 119 in the rich medium condition. In medium containing a variety of carbon sources, increased growth rate and survival likely depends on utilizing multiple carbon sources simultaneously, thus requiring the additional components involved. In conditions were carbon is limited to a sole source, these additional components become a burden to the cell; requiring resources, but granting no benefit.

Establishing the conditional contribution of each gene under rich and minimal media conditions establishes Tn-seq as a potential tool for the study of *M.*, subterrani as

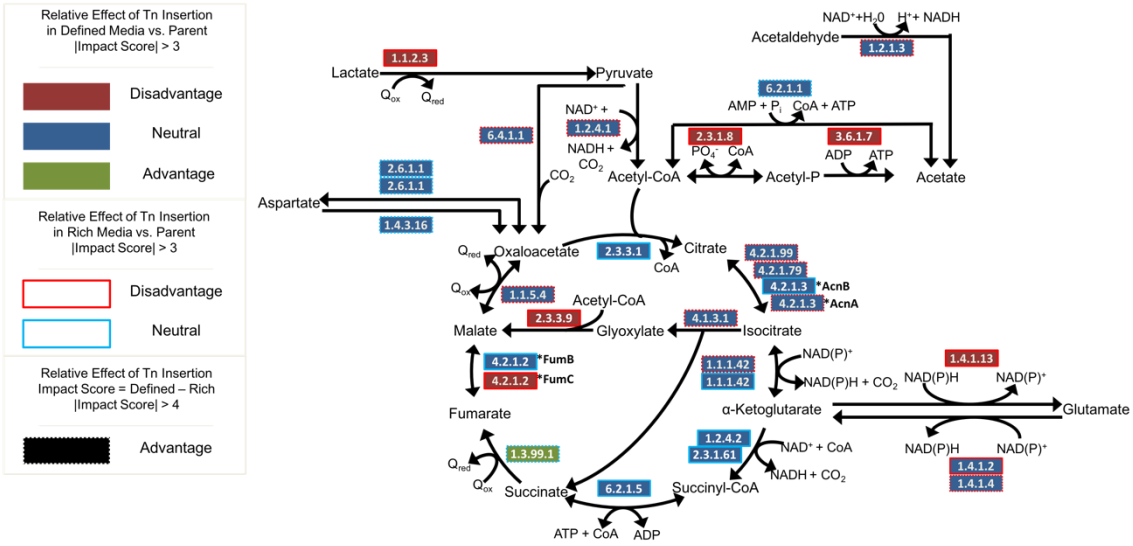


Figure 4.3. Metabolic map representing relative growth contribution of genes with products involved in the citrate cycle. Impact score (IS) is described by IS=ln(mrc/mrp) where mrc is the normalized reads from the condition mapping to a gene, and mrp is the reads mapping to the same gene from the parental population.

well as providing a basis for building a metabolic framework for *M. subterrani*. The primary source of carbon and electrons under both conditions centralize around the tricarboxylic acid cycle (Figure 4.3). Amino acids provide the primary source of both fixed carbon and electrons in LBMB medium for heterotrophic growth, with a relative abundance of glutamate. Disruption of glutamate dehydrogenases (E.C. 1.4.1.2 and 1.4.1.4) have a negative impact on growth, presumably as glutamate is fed into the TCA cycle at α-ketoglutarate. However, similar disruptions under minimal conditions do not. As lactate is the sole source of carbon in MIM, flux drives the reduction of α-ketoglutarate towards the production of glutamate through glutamate synthase (E.C. 1.4.1.13). This is further supported by the neutral effect of disruptions in glutamate dehydrogenases (E.C. 1.4.1.2 and 1.4.1.4), as interconversion would provide little benefit. In addition to providing information by suggesting flux through metabolism, Tn-seq results may also suggest roles for analogous enzymes. Transposon mutants disrupting

fumarases produce similar growth effects between rich and minimal media conditions. *M. subterrani* encodes two putative fumarase genes, *fumB* and *fumC*. In *E. coli fumB* is upregulated during anaerobic growth (Tseng, 1997; Woods et al., 1988), whereas *fumC* is upregulated during iron starvation or oxidative stress and repressed anaerobically (Park and Gunsalus, 1995). The neutral effect on *fumB*, but deleterious effect on *fumC* is expected as cultures were grown aerobically. However, the presence of *fumC*, but absence of a *fumA*, may suggest *M. subterrani* is accustomed to oxidative stress (Park and Gunsalus, 1995).

DEATH-seq

Following a 72-hour incubation in unsterilized mine water, total *M. subterrani* cell density decreased from 3.6×10^7 CFU/mL to 3.1×10^5 CFU/mL. Half the culture was processed for DEATH-seq analysis (T1). The remaining culture was pelleted and resuspended in 50 mL of LBMB and grown from 3.2×10^6 CFU/mL to 1.8×10^7 CFU/mL (T2). A threshold of 10^3 reads mapped to a gene in either the parent or the conditional library was considered sufficient for analysis, while 10^2 reads mapped was the threshold for being represented in the library. Of the 3,272 genes in the T1 library with sufficient reads mapped, a disruption in 1,352 resulted in relatively decreased survival. The library T2 contained 3,264 passing genes, of which 1,392 resulted in relatively decreased survival. Libraries represented largely the same genes, with only 19 genes found in a single library only. However, 714 genes switched between beneficial and detrimental from T1 to T2, suggesting outgrowth of the initial conditional library

alters relative abundance of mapped reads, but does not drastically alter library composition. Altered relative abundance is expected, as genes contributing to rapid growth under nutrient replete conditions are unlikely to have similar benefit during starvation stress. Though when only the 10% most deleterious and 10% most beneficial genes were considered for T1, only 40 genes switched during outgrowth to T2, and only 12 of those were of a magnitude to display as significant false positives in the T2 dataset. Attempts to rectify discrepancies by normalizing with the data obtained in the Tn-seq LBMB condition were unsuccessful. It is suggested if outgrowth is required due to experimental design, that doublings be minimalized.

Though no clear pattern emerges from either T1 or T2 in regards to survival in unfiltered mine water, several considerations from the results would benefit future work on a high throughput transposon analysis involving persistence as the selection. Assaying persistence phenotypes will inherently result in a decreased overall population during selection, thus obtaining sufficient genetic material may be difficult in conditions where starting cell number is already limited. In these cases, where the surviving population is outgrown to obtain sufficient biomass for processing, mutant effects on outgrowth must also be considered. Additionally, the use of this technique with high cell density starting populations warrants caution towards use in oligotrophic organisms. Though catabolism of deceased cells under survival conditions resulting in growth is often unavoidable, it is difficult to distinguish a positive final score resulting from growth, survival, or as an artifact from outgrowth; as each requires different normalization from the starting population.

Comparing Essential Gene Profiles

The six strains of *Marinobacter* used in this study share a core genome of 1860 genes, with a pan genome of 6361 genes. Transposon mutant libraries were generated in six strains of *Marinobacter* and the insertion frequencies determined by Illumina sequencing (Summarized in Table 4.3). Genes containing no mapped reads were deemed essential as transposon disruption should prove lethal at these loci and will not be represented in the library. GET_HOMOLOGUES based comparison of the essential gene profiles suggests an essential core genome of only 43 genes, with 1933 in the essential pangenome. Such a small set of shared essential genes is unlikely given the number of genes required to perform universal functions and the size of the core genome. Cursory manual curation based on automated annotation suggests the essential core genome is indeed larger. Putative errors in genome construction, differences in open reading frame estimation, and essentiality definition all contributed to an underestimation in the

Table 4.3. Transposon Mutant Library Sequencing and Mapping Results

		1	2	3	4	5	6
Genome size	**Mb**	4.45	4.77	4.76	4.39	3.58	3.99
ORFS	**Total**	4224	4489	4458	4111	3363	3860
	Mapped	3592	3916	3906	3201	2821	2844
	%	85.04	87.24	87.62	77.86	83.88	73.68
Reads	**Total**	70M	19.31M	19.53M	16.7M	21.92M	18.9M
	Mapped	6.06M	6.51M	6.29M	5.50M	7.58M	5.47M
	%	8.67	33.75	32.25	32.98	34.59	28.95
TA sites	**Total**	106072	125537	116200	110262	90460	105519
	Mapped	54185	61557	84353	32387	45789	30431
	%	51.08	49.03	72.59	29.37	50.62	28.84
Essentials	**# of genes**	632	573	552	910	542	1016
	%	14.96	12.76	12.38	22.14	16.12	26.32

1. *Marinobacter subterrani JG233*
2. *Marinobacter* sp. CP1
3. *Marinobacter* sp. JG228
4. *Marinobacter*sp. FeSN6p
5. *Marinobacter persicus* UTICA-S1B3
6. *Marinobacter hydrocarbonoclasticus* ATCC 49840

essential core genome. For example, automated selection of essential gene homologues estimated the small ribosomal protein S19p to be essential in all the organisms assayed with the exception of *M. subterrani*. However, due to a putative A to G sequencing error in the start codon this gene is annotated as truncated and is therefore not included in the orthologue cluster, but rather is deemed essential as a separate cluster. Manual curation entailing assessment of the unique nature of each cluster individually is required to fully assay the effectiveness of this technique and validity of the data. Clusters containing orthologous sequences will be consolidated, decreasing the overall variance in the essential pangenome, while increasing the essential core genome to better represent core essential functions in the genus *Marinobacter*.

The use of transposon mutagenesis to describe genetic contribution to survival and growth demonstrate the advanced molecular techniques available for the interrogation of the genus *Marinobacter*, as well as providing a foundational understanding of *M. subterrani* growth under heterotrophic conditions. As the collective metabolic versatility and range of environmental tolerance of the *Marinobacter* continue to expand with each additional isolate, the biotechnological promise of this genus will drive the refinement and sustained development of the techniques available.

Chapter 5: Comparative Genomics of the Genus *Marinobacter*

Summary

As techniques, technology, and understanding advance, relegated data harbors new potential. The ubiquity and ease by which members of the genus *Marinobacter* are propagated results in an abundance of publically available data that has been largely dismissed after initial use. However, the *Marinobacter* are capable of thriving in extreme environments and employ diverse metabolic capabilities, and thus their study may yield biotechnologically valuable insights and tools. Comparison of *Marinobacter* strains using average nucleotide and amino acid identity suggest simplification of genus taxonomy through assignment and reassignment of species level classification. Additionally, comparative genomics of strains grouped by trait suggests factors involved in conditional tolerance and biome preference.

Introduction

Bacteria of the genus *Marinobacter* are found ubiquitously in aquatic and terrestrial salinous environments. Gram-negative and generally heterotrophic bacilli, the genus displays a remarkable diversity regarding metabolic capacity, habitat, and conditional tolerance. *Marinobacter lutaoensis* is capable of growth at 50^{o}C, while *Marinobacter subterrani* grows at temperatures approaching freezing (Shieh et al., 2003; Bonis and Gralnick, 2015). Several strains are capable of growth in salinities ranging from negligible to 20.5%, and are quickly acclimated to sodium chloride concentrations high as 35% (Gauthier et al., 1992; Handley and Lloyd, 2013). Recalcitrance to environmental shifts, coupled with broad utilization of carbon sources, has prompted development of *Marinobacter* species as industrially relevant hosts and biocatalysts. In addition to

phenotypic variation, the *Marinobacter* harbor a great deal of genomic diversity as well; with genome sizes ranging from 2.5-5.4Mb and GC-content varying from 53.7-63.2%. Belying the tremendous variation in phenotypic traits, members of the genus *Marinobacter* share high identity by 16S rDNA, which would traditionally suggest members have recently diverged as species, and thus have similar genomic content. Species isolated from Lake Vida, a perennially ice-covered lake in Antarctica, share high 16S rDNA identity with those found more equatorially, despite have been isolated for at least 2,800 years in an extremely selecting environment (Kuhn et al., 2014).

Access to tremendous amounts of data has accompanied the advent of high throughput sequencing technology. Complete genomes have become incidental findings of metagenomics studies and sequencing of isolate genomes has become standard. The universal nature of the *Marinobacter* and the ease by which they are cultivated has led to a vast increase in public repository sequencing data. Unfettered by the limitations of comparing organisms by short stretches of genomic sequence enables revised taxonomic analysis using modernized and informed approaches, as well as a means of refining and directing studies into the shared and defining features of clades. Environmentally influential, biochemically diverse, and industrially relevant, the *Marinobacter* represent a relatively uncharacterized and unexplored biological resource. Using 71 quality genomes acquired from public and private collections, we describe full genome comparison across phenotypic and phylogenetic groups. Focusing on revising taxonomic classification and genotypic markers contributing to conditional tolerance to salinity, temperature, and biome of isolation.

Materials and Methods

Bioinformatic analysis

Publically available genomes were acquired from the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and the Joint Genome Institute's Integrated Microbial Genomes website (www.img.jgi.doe.gov). Private genomes were sequenced using a combination of Illumina, Pacific Biosciences SMRT, and Oxford Nanopore Technologies MinION sequencing platforms. The Rapid Annotation using Subsystem Technology platform (http://rast.nmpdr.org/) was used for gene calling and annotation for strains with this information not publically available. Strains, accession numbers, and genome parameters are outlined in Table 5.1. Pangenome analysis was performed using the GET_HOMOLOGUES (Contreras-Moreira and Vinuesa, 2013) software package and the Anvi'o analysis platform (Eren et al., 2015). Basic Local Alignment Search Tool (BLAST) was used to construct the matrix in GET_HOMOLOGUES and clustering was performed using OrthoMCL (Li et al., 2003) with Pfam with a minimum sequence identity of 30%. Average nucleotide identity and average amino acid identity were calculated using the ANI/AAI-Matrix: All-vs-all ANI/AAI matrix calculator (Rodriguez and Konstantinidis, 2016). Literature values were used for biome of calculated using the ANI/AAI-Matrix: All-vs-all ANI/AAI matrix calculator (Rodriguez and Konstantinidis, 2016). Literature values were used for biome of isolation and tolerance calculated using the ANI/AAI-Matrix: All-vs-all ANI/AAI matrix calculator (Rodriguez and Konstantinidis, 2016). Literature values were used for biome of isolation and tolerance parameters for minimum, maximum, and optimum tolerance for

Table 5.1. *Marinobacter* strains.

species	strain	database	accession(s)	contigs	total length (bp)	total CDS	Notes
sp.	AC-23	NCBI	NZ_MBPP00000000	105	4149715	3128	Kongsfizorden sediment
adhaerens	HP15	NCBI	CP001978- CP001980.1	3	4651725	4410	symbiont
adhaerens	PBVC038	NCBI	NZ_LXRF00000000.1	21	4246510	3847	symbiont
algicola	DG893	NCBI	ABCP01000001.1	104	4412523	4127	Yellow Sea
antarcticus	CGMCC 1.10835	IMG	Ga0079884	11	3740786	3481	Antartic sand
daepoensis	DSM16072	IMG	Ga0002817	17	3835778	3530	Yellow Sea
daqiaonensis	CGMCC 1.9167	IMG	Ga0079877	8	3933786	3544	Yellow Sea saltern
excellens	HL-55	NCBI	LJZQ01000001.1	55	3782657	3499	Hot Lake washington
excellens	LAMA 842	NCBI	LOCO01000001.1	59	4377629	3984	lab enrichments
sp.	FeSN1--63	N/A	this study	41	4369315	4106	marine sediment
sp.	FeSN1p--87	N/A	this study	67	4280244	4111	marine sediment
sp.	FeSN6p--85	N/A	this study	53	4391972	4111	marine sediment
sp.	FeSNey--86	N/A	this study	47	4388763	4117	marine sediment
gudaonensis	CGMCC 1.6294	IMG	Ga0070159	9	3839018	3441	salt pond
hydrocarbonoclasticus	ATCC49840	NCBI	FO203363.1	1	3989480	3804	oil polluted seawater
hydrocarbonoclasticus	VT8	NCBI	CP000514.1-CP000516.1	3	4779762	4272	oil polluted seawater
lipolyticus	BF04_CF-4	IMG	Ga0025431	139	3633797	3753	Blood Falls, Antarctica
lipolyticus	SM19	NCBI	ASAD01000001.1	43	4023208	3627	saline soil
lutaoensis	T5054	NCBI	NZ_MSCW00000000.1	25	3827309	3463	hotspring/marine mixing
manganoxydans	MnI7-9	NCBI	AGTR01000001.1	88	4549590	4203	deep sea
mobilis	CGMCC 1.7059	IMG	Ga0070178	26	3971609	3603	East China Sea
nanhaiticus	D15-8W	NCBI	APLQ00000000.1	14	5358309	4765	South China Sea
nitratireducens	AK21	NCBI	ANIE01000001.1	22	3888776	3523	surface ocean
pelagius	CGMCC 1.6775	IMG	Ga0070169	16	3800146	3499	China Sea
persicus	IBRC-M 10445	IMG	Ga0104431	24	3158780	2916	Hypersaline lake
persicus	SH-1B Col3	IMG	2700989663	94	3577165	3356	Frack Fluid
persicus	SH-1B Col6	IMG	2700989662	96	3581634	3363	Frack Fluid
persicus	SH-1B Col9	IMG	2700989665	100	3579257	3357	Frack Fluid
psychrophilus	20041	NCBI	CP011494.1	1	3998597	3450	Arctic ice blocks

species	strain	database	accession(s)	contigs	total length (bp)	total CDS	Notes
salarius	R9SW1	NCBI	CP007152.1	1	4616532	3168	Sea of Japan
salinus	N/A	NCBI	NZ_CP017715.1	1	4121005	3869	tidal flat
salsuginis	SD-14B	IMG	Ga0011358	369	4733264	4702	brine deeps (red sea)
santoriniensis	NKSG1	NCBI	APAT01000001.1	41	4033468	3693	hydrothermal sediments
segnicrescens	CGMCC 1.6489	IMG	Ga0070163	85	4218891	3897	South China Sea
similis	A3d10	NCBI	CP007151.1	1	3975896	2692	Sea of Japan
sp.	BSs20148	NCBI	CP003735.1	1	4063864	3887	Canadian basin
sp.	C1S70	NCBI	AXBW01000001.1	105	4122849	3825	Deepwater Horizon
sp.	CP1	NCBI	CP011929.1	1	4768422	4173	Sediment Enrichment
sp.	DS40M8	IMG	Ga0011388	795	2506529	2842	marine
sp.	DSM 26291	IMG	Ga0070016	53	4492204	4121	Gulf of Mexico
sp.	DSM 26671	IMG	Ga0070017	77	4867885	4418	Gulf of Mexico
sp.	ELB17	NCBI	AAXY01000001.1	75	4894744	4850	Antarctic (lake bonney)
sp.	EN3	NCBI	AXCC01000001.1	106	3975756	3697	Deepwater Horizon
sp.	ES-1	NCBI	AXBV01000001.1	117	3556390	3319	Deepwater Horizon
sp.	ES.042	IMG	Ga0070022	1	3936136	3579	intertidal microbial mat
sp.	ES.048	IMG	Ga0070024	2	4063301	3750	intertidal microbial mat
sp.	EVN1	NCBI	AXCB01000001.1	71	4297263	4000	Deepwater Horizon
sp.	Hex_13	NCBI	LSMM01000001.1	95	4243535	3757	Deepwater Horizon
sp.	HL-58	NCBI	LIHP01000001.1	21	4466715	4071	Hot lake washington
sp.	JG228	N/A	this study	1	4763635	4408	Soudan Mine
sp.	LQ44	NCBI	CP014754.1	1	4435564	3997	hydrothermal sediments
sp.	LV10MA510-1	IMG	Ga0070630	1	4447234	4330	Antarctica (Lake Vida)
sp.	LV10R510-11A	IMG	Ga0070626	1	4436489	4288	Antarctica (Lake Vida)
sp.	LV10R510-8	IMG	Ga0070629	1	4333333	4233	Antarctica (Lake Vida)
sp.	LV10R520-4	IMG	Ga0070250	1	4421958	4256	Antarctica (Lake Vida)
sp.	LVR2A5A20	IMG	Ga0070251	1	4663413	4531	Antarctica (Lake Vida)
sp.	MCTG268	IMG	Ga0004723	14	4449396	4083	symbiont
sp.	REDSEA-S15_B16	IMG	Ga0114055	527	2916776	3435	derived from metagenome
sp.	REDSEA-S21_B2N3	IMG	Ga0114056	262	4386380	4266	derived from metagenome

species	strain	database	accession(s)	contigs	total length (bp)	total CDS	Notes
sp.	REDSEA-S27_B10	IMG	Ga0114057	500	3337778	3727	derived from metagenome
sp.	tcs11	NCBI	LLYU01000001.1	51	4266557	3925	Red Sea
sp.	YWL01	NCBI	LORG01000001.1	965	4538697	4299	annotated manually
sp.	C18	NCBI	GCA_001858325.1	42	4934532	4461	symbiont
sp.	EhC06	NCBI	GCA_001650915.1	38	4620180	4142	symbiont
sp.	EhN04	NCBI	GCA_001650765.1	38	4619005	4138	symbiont
sp.	T13-3	NCBI	GCA_001577575.1	1265	4266557	3925	Frack Fluid
sp.	X15-166B	NCBI	GCA_001752365.1	27	3488334	3386	Hawai'i Harbor
subterrani	JG233	NCBI	LFBU01000001.1	2	4453613	4152	Soudan Mine
vinifirmis	P4B1	NCBI	LLXM01000001.1	15	3740877	3373	marine sediment
vinifirmus	FB1	NCBI	NZ_NEFY00000000	60	3836576	3475	Vineyard Waste
zhejiangensis	CGMCC 1.7061	IMG	Ga0070179	12	4029256	3660	East China Sea

temperature, salinity, acidity (Table 5.2). Moderate tolerance groupings are defined as within a single standard deviation from the mean, while low and high grouping were below and above a single standard deviation from the mean, respectively. Strains from Antarctic environments without published biochemical characterization were considered as low temperature organisms.

Results and Discussion

Taxonomic Analysis

Despite recent advances in sequencing and the surfeit of accessible genomic data, many organisms remain classified by single gene comparison and tedious physiological analysis, leading many organisms to remain unclassified to the species level. Bacterial nomenclature has, by necessity, been plastic as our technology and understanding of bacterial relatedness advances. Originally described as a novel species, *Marinobacter aquaeolei* VT8 was reclassified under *Marinobacter hydrocarbonoclasticus* as DNA-DNA hybridization became a superior method of speciation (Márquez and Ventosa, 2005). Using public and private genomes and comparison by average nucleotide (ANI) and amino acid identity (AAI) and the percent identity cutoffs defined by Rodriguz and Konstantinidis, 2014, we propose new taxonomic classification to members of the genus *Marinobacter* (Table 5.3). In addition to *Marinobacter hydrocarbonoclasticus* VT8, we suggest spp. C1S70, EN3, EVN1, and tcs11 be included as strains of *Marinobacter hydrocarbonoclasticus* based on agreeing ANI and AAI values above the species thresholds of 95% and 90%, respectively.

Table 5.2. Tolerance and biome groupings of *Marinobacter* based on literature values.

Optimum Salinity Tolerance
High
M. daqiaonensis CGMCC1.9167
M. lipolyticus SM19
M. persicus IBRC-M10445
M. santoriniensis NKSG1
M. segnicrescens CGMCC1.6489
M. subterrani JG233
Moderate
M. adhaerens HP15
M. algicola DG893
M. antarcticus CGMCC1.10835
M. daepoensis DSM16072
M. hydrocarbonoclasticus VT8
M. mobilis CGMCC1.7059
M. nanhaiticus D15-8W
M. pelagius CGMCC1.6775
M. salsuginis SD-14B
M. hydrocarbonoclasticus ATCC49840
M. sp. ELB17
Low
M. gudaonensis CGMCC1.6294
M. nitratireducens AK21
M. zhejiangensis CGMCC1.7061

Biome of Isolation
Terrestrial
M. lipolyticus SM19
M. persicus SH-1B Col3
M. persicus SH-1B Col6
M. persicus SH-1B Col9
M. sp. JG228
M. sp. T13-3
M. subterrani JG233
Aquatic
M. lutaoensis
M. salinus Hb8
M. sp. ELB17
M. sp. LV10MA510-1
M. sp. LV10R510-11A
M. sp. LV10R510-8
M. sp. LV10R520-4
M. sp. LVR2A5A20

Biome of Isolation (continued)
Marine
M. adhaerens HP15
M. adhaerens PBVC038
M. algicola DG893
M. antarcticus CGMCC1.10835
M. daepoensis DSM16072
M. daqiaonensis CGMCC1.9167
M. excellens LAMA842
M. sp. FeSN1
M. sp. FeSN1p
M. sp. FeSN6p
M. sp. FeSNey
M. gudaonensis CGMCC1.6294
M. hydrocarbonoclasticus ATCC49840
M. hydrocarbonoclasticus VT8
M. manganoxydans MnI7-9
M. mobilis CGMCC1.7059
M. nanhaiticus D15-8W
M. nitratireducens AK21
M. pelagius CGMCC1.6775
M. persicus IBRC-M10445
M. psychrophilus 20041
M. salarius R9SW1
M. salinus Hb8
M. salsuginis SD-14B
M. santoriniensis NKSG1
M. segnicrescens CGMCC1.6489
M. similis A3d10
M. sp. AC-23
M. sp. BSs20148
M. sp. C18
M. sp. C1S70
M. sp. CP1
M. sp. DS40M8
M. sp. DSM26291
M. sp. DSM26671
M. sp. EhC06
M. sp. EhN04
M. sp. EN3
M. sp. ES-1
M. sp. EVN1
M. sp. Hex 13
M. sp. MCTG268
M. vinifirmus P4B1
M. sp. REDSEA-S15 B16
M. sp. REDSEA-S21 B2N3
M. sp. REDSEA-S27 B10
M. sp. tcs11
M. sp. X15-166B
M. sp. YWL01
M. zhejiangensis CGMCC1.7061

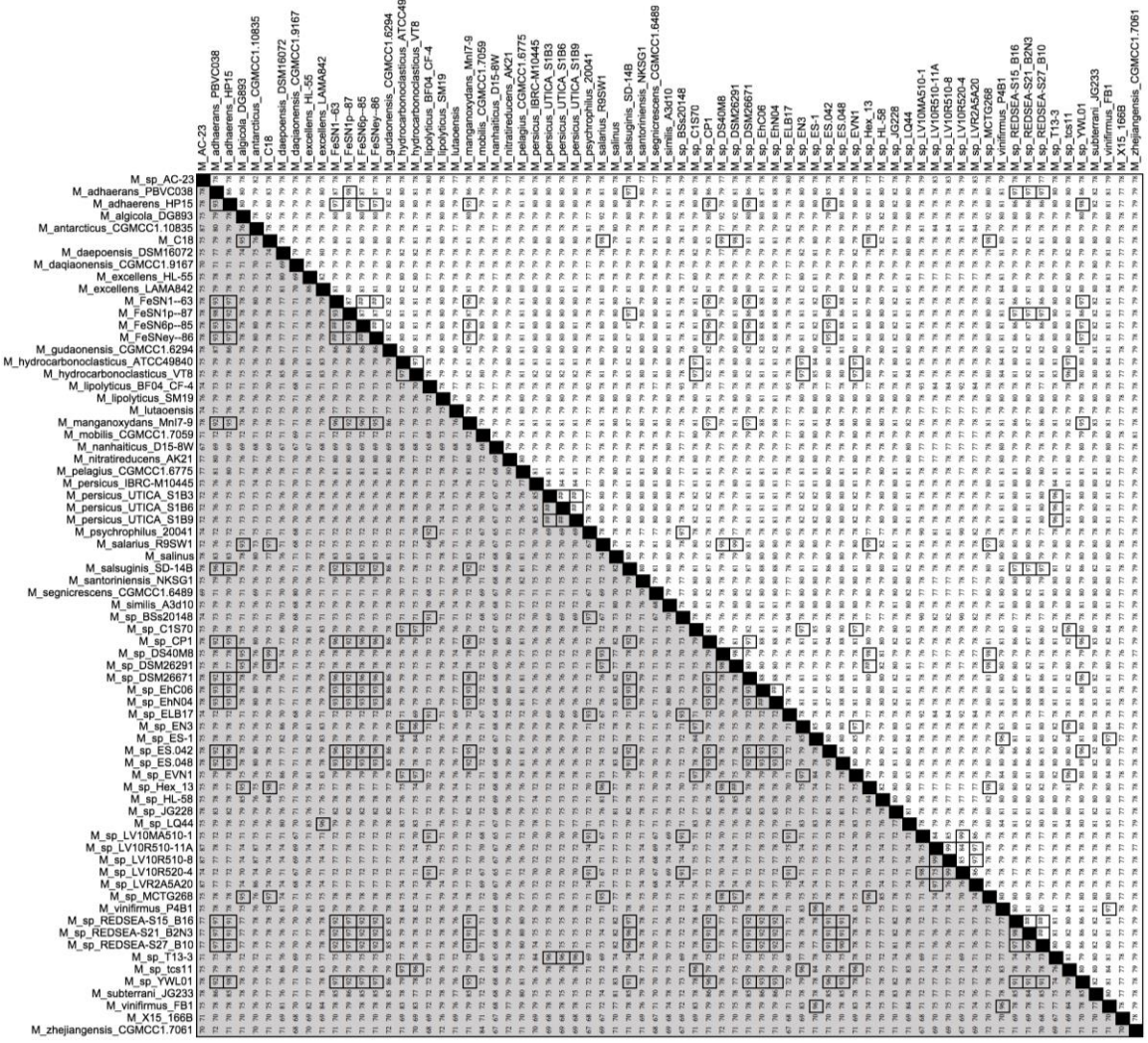
Maximum Temperature Tolerance
High
M. hydrocarbonoclasticus VT8
M. pelagius CGMCC1.6775
M. lutaoensis
M. santoriniensis NKSG1
Moderate
M. adhaerens HP15
M. algicola DG893
M. excellens LAMA842
M. lipolyticus SM19
M. mobilis CGMCC1.7059
M. nanhaiticus D15-8W
M. nitratireducens AK21
M. salarius R9SW1
M. santoriniensis NKSG1
M. similis A3d10
M. subterrani JG233
M. zhejiangensis CGMCC1.7061
M. daepoensis DSM16072
M. daqiaonensis CGMCC1.9167
M. gudaonensis CGMCC1.6294
M. hydrocarbonoclasticus ATCC49840
M. persicus IBRC-M10445
M. salsuginis SD-14B
M. segnicrescens CGMCC1.6489
Low
M. antarcticus CGMCC1.10835
M. lipolyticus BF04 CF-4
M. psychrophilus 20041
M. sp. AC-23
M. sp. BSs20148
M. sp. ELB17
M. sp. LV10MA510-1
M. sp. LV10R510-11A
M. sp. LV10R510-8
M. sp. LV10R520-4
M. sp. LVR2A5A20

Maximum Salinity Tolerance
High
M. antarcticus CGMCC1.10835
M. hydrocarbonoclasticus ATCC49840
Moderate
M. daepoensis DSM16072
M. daqiaonensis CGMCC1.9167
M. excellens LAMA842
M. gudaonensis CGMCC1.6294
M. lipolyticus SM19
M. nanhaiticus D15-8W
M. pelagius CGMCC1.6775
M. segnicrescens CGMCC1.6489
M. adhaerens HP15
M. hydrocarbonoclasticus VT8
M. persicus IBRC-M10445
M. salarius R9SW1
M. salsuginis SD-14B
M. santoriniensis NKSG1
M. similis A3d10
M. algicola DG893
M. subterrani JG233
Low
M. mobilis CGMCC1.7059
M. nitratireducens AK21
M. psychrophilus 20041
M. zhejiangensis CGMCC1.7061

Despite sharing 99% identity of the 16rRNA gene, *Marinobacter adhaerens* and unclassified species bin into two distinct clusters by ANI with a species cutoff of 95%. The first, containing the type strain *Marinobacter adhaerens* HP15, also includes *Marinobacter manganoxydans* Mnl7-9, and spp. FeSN1, FeSN6p, FeSNey, CP1, DSM26671, ES.042, and YWL01. The second distinct cluster groups with *Marinobacter adhaerens* PBVC038, and includes *Marinobacter salsuginis* SD-14B(T) and spp. FeSN1p, REDSEA-S15_B16, REDSEA-S21_B2N3, and REDSEA-S27_B10. The high AAI values suggest these two clades may encounter similar selective pressure, but have diverged sufficiently by silent mutations to constitute two separate species. Given chronological deference and characterization of the type strain HP15, we suggest *Marinobacter manganoxydans* Mnl7-9 be renamed to *Marinobacter adhaerens* Mnl7-9, and the spp. FeSN1, FeSN6p, FeSNey, CP1, DSM26671, ES.042, and YWL01 be classified as *Marinobacter adhaerens* strains. The *Marinobacter adhaerens* PBVC038 clade would be identified as *Marinobacter salsuginis*, of which strain SD-14B(T) would be the type strain, and include stains PBVC038, FeSN1p, REDSEA-S15_B16, REDSEA-S21_B2N3, and REDSEA-S27_B10.

Marinobacter lipolyticus contains two strains, SM19 and BF04_CF-4, that share 95% identity of the 16S rRNA gene. Though this falls below the current understanding requiring a greater than 98.2% identity, ANI and AAI results confirm that *Marinobacter lipolyticus* SM19 and BF04_CF-4 are two separate species. It is suggested *Marinobacter lipolyticus* BF04_CF-4 be renamed *Marinobacter psychrophilis* BF04_CF-4 based on ANI and AAI similarity to the type strain *Marinobacter psychrophilis* 20041(T). *Marinobacter psychrophilis* would also include new strains BSs20148 and ELB17.

Table 5.3. ANI and AAI of *Marinobacter* strains.



*Shaded boxes indicate Average Amino Acid Identity, while white boxes indicate Average Nucleotide Identity. Bordered values indicate values at, or above, the species threshold proposed by Rodriguz and Konstantinidis, 2014.

Marinobacter salarius R9SW1 was assigned a new species designation from *Marinobacter algicola* LMG 23835 based on biochemical differences in electron accepter and donor utilization, despite an over 99% 16S rRNA gene identity (Ng et al, 2014). However, the *Marinobacter* display a remarkable diversity in biochemical traits and

metabolic capabilities; suggesting such accessory genetic elements are quickly lost or gained, and thus make poor candidates for discriminating classification. Similar ANI percentages and greater than 90% AAI percent support the inclusion of *Marinobacter salarius* R9SW1 under *Marinobacter algicola*. In addition to the former *Marinobacter salarius* R9SW1, we propose *Marinobacter algicola* include new strains C18, DS40M8, DSM26291, Hex-13, and MCTG268 based on ANI and AAI comparison.

Although the *Marinobacter persicus* strains share a 99% or greater 16S rRNA gene sequence identity, the ANI and AAI values do not support the inclusion under a single species designation. We propose the type strain, *Marinobacter persicus* IBRC-M10445, maintain the species name, but strains UTICA_S1B3, UTICA_S1B6, UTICA_S1B9 be reclassified under a new species name that additionally includes the sp. T13-3. Finally, we propose *Marinobacter vinifirmus* to include the new strain ES-1 in addition to P4B1 and the type strain FB1.

Comparative Genomics

Genes defining *Marinobacter* based on extremity tolerances to temperature, salinity, acidity, or biome of isolation were determined by inclusion in every member of one group to the full exclusion of the other group (summarized in Table 5.4). Strains within one standard deviation regarding each respective trait were not compared to high and low groups; as conditional tolerance results from a progressive evolution, this inherent multivariance complicates clear delineation from the more extreme groups. Comparison of isolates of high or low maximum temperature tolerance yielded 105 genes; 89 found in high temperature isolates and 16 in the lower. A minimum of 16 genes found in higher

temperature isolates putatively encode products involved in membrane and cell wall maintenance, though an additional 2 hypothetical genes are likely involved as well due to co-localization and synteny across the strains assayed. An additional 5 putatively encode proteins involved in stress shock may also contribute to temperature tolerance. The remaining annotated genes categorize into nitrogen metabolism, utilization of small organic acids, and gene regulation. Of the 16 genes found in the lower temperature isolates, the majority are poorly annotated and thus make interpretation difficult. It is worth noting only four genes in the entire analysis were found in an extremity cluster to the full exclusion of the moderate group; two in the high maximum temperature and two in the low maximum temperature groups, though these genes lack any predicted function. Comparison of the high and low maximum salinity tolerance groups yielded 48 genes, of which 38 were found in the high tolerance group. These trended towards regulation and membrane transport, while no trend can be found in the low tolerance genes. The remaining tolerance groupings displayed no genes potentially diagnostic to the grouping. The lack of clear delineation by gene content between groupings may be primarily due to differential activity of common genes and regulation, though it is more likely due to multivariate phenotypes which are acquired progressively. Analysis accounting for the contribution of individual genes to cumulative phenotype would likely yield better results. It is worth noting, though not to the exclusion of marine strains, terrestrial isolates exclusively use urea carboxylase, and none have been found to encode a urease. Genome scale comparison of the 71 sequenced *Marinobacter* with relatedness determined by tetranucleotide frequency suggests psychrophilic strains diverged early from the rest of the genus (Figure 5.1). This psychrophilic clade is further defined by an

Table 5.4. Orthologous clusters exclusive to *Marinobacter* groupings.

Maximum Temperature Tolerance

High exclusive of Low

1-acyl-sn-glycerol-3-phosphate acyltransferase
2,3-diketo-5-methylthio-1-phosphopentane phosphatase
23S rRNA (cytidine2498-2'-O)-methyltransferase
ABC transporter [3]
acetolactate synthase-1/2/3 large subunit
Activator of Hsp90 ATPase 1 family protein
Biopolymer transport protein ExbD [2]
C4-dicarboxylate ABC transporter
C4-dicarboxylate transport sensor histidine kinase DctB
cation transport regulator ChaB
Co/Zn/Cd efflux system
coprogen and ferric-rhodotorulic acid
cspV cold-shock DNA-binding protein family
cyclohexanone monooxygenase
cysteine synthase A
Dicarboxylate transport
diguanylate cyclase
dihydrolipoamide acetyltransferase
ectoine hydroxylase
exopolyphosphatase
exosortase
Exporter of O-antigen and teichoic acid
FhuF 2Fe-2S C-terminal domain-containing protein
glnD (Nitrogen regulation)
Glycosyltransferase
Glycosyltransferase involved in cell wall bisynthesis [3]
Glyoxylase, beta-lactamase superfamily II
GntR family transcriptional regulator
haloalkane dehalogenase
heat shock protein Hsp20
histidine kinase
hypothetical [17]
L-lactate dehydrogenase complex protein LldE
L-lactate dehydrogenase complex protein LldG
metal dependent phosphohydrolase
MFS transporter
mgsA methylglyoxal synthase
MoxR-like ATPase (chaperone)
MSHA biogenesis protein MshI, fimbria assembly
Na/Pi cotransporter
NAD(P)H dehydrogenase (quinone)
nitrogen regulatory protein P-II
O-antigen ligase
outer membrane transport energization protein ExbB
PEP-CTERM system associated sugar transferase
PEP-CTERM system associated, cell wall protein
Phage integrase, N-terminal SAM-like domain
phenylacetate-CoA ligase
Polysaccharide deacetylase
Predicted thiol-disulfide oxidoreductase YuxK, DCC family
Cystathionine beta-synthase, core domain
Protein-S-isoprenylcysteine O-methyltransferase Ste14
RNA polymerase sigma-70 factor, ECF subfamily
RNA-binding protein, contains PUA-like domain
rRNA pseudouridine516 synthase
Small-conductance mechanosensitive ion channel
sodium/proton antiporter
sulfoxide reductase catalytic subunit YedY
sulfoxide reductase heme-binding subunit YedZ
thiamine binding protein
thioredoxin/CopG transcriptional regulator
transcriptional regulator, AraC family
transposase
tRNA (guanosine-2'-O-)-methyltransferase
tRNA(Met)-cytidine N(4)-acetyltransferase
tRNA/rRNA methyltransferase
tryptophan synthase beta chain
UDP-2,3-diacylglucosamine pyrophosphatase LpxH
UDP-N-acetylglucosamine pyrophosphorylase

Low exclusive of High

16S rRNA pseudouridine516 synthase
DNA-binding response regulator, OmpR family
exopolyphosphatase
FAD-dependent dehydrogenases
Fe-S oxidoreductase
HNH endonuclease
hypotheical [6]
NADH:flavin oxidoreductases, Old Yellow Enzyme family
protease
RIO kinase 1
RND family efflux transporter, MFP subunit
signal transduction histidine kinase
tol-pal system beta propeller repeat protein TolB

Maximum Salinity Tolerance

High exclusive of Low	**Low exclusive of High**
4-mercaptohistidine N1-methyltranferase	CobN
Acyltransferase [2]	flagellar assembly protein FliH
Cytochrome C	formate dehydrogenase accessory protein FdhD
Diguanylate cyclase	hypothetical [2]
Ferritin	Isochorismatase
hypothetical [11]	methylmalonate-semialdehyde dehydrogenase
Integrase	Predicted lipid carrier protein YhbT
Metallophosphoesterase	ribonucleoside-diphosphate reductase alpha chain
Methyl-accepting chemotaxis protein [3]	Transcriptional regulators of sugar metabolism
NAD/FAD-dependent oxidoreductase	
NADPH-dependent FMN reductase	**Optimum Salinity**
Permease [3]	**Low exclusive of High**
putative 4-mercaptohistidine N1-methyltranferase	RNA polymerase sigma-70 factor, ECF subfamily
Response regulator	tRNA(Met)-cytidine N(4)-acetyltransferase
Rhodanese-like sulfurtransferase	hydrolase
RNA polymerase nonessential primary-like sigma factor	hypothetical
Sensors of blue-light using FAD	
Signal transduction histidine kinase	**Biome of Isolation**
Spermidine synthase	**Absent exclusively in Terrestrial**
Tat pathway signal protein	ureA
Transporter	ureF urease accessory protein
Transposase	ureG urease accessory protein
TrkA-C domain-containing protein	

*Bracketed numbers indicate a tally of genes with identical annotation

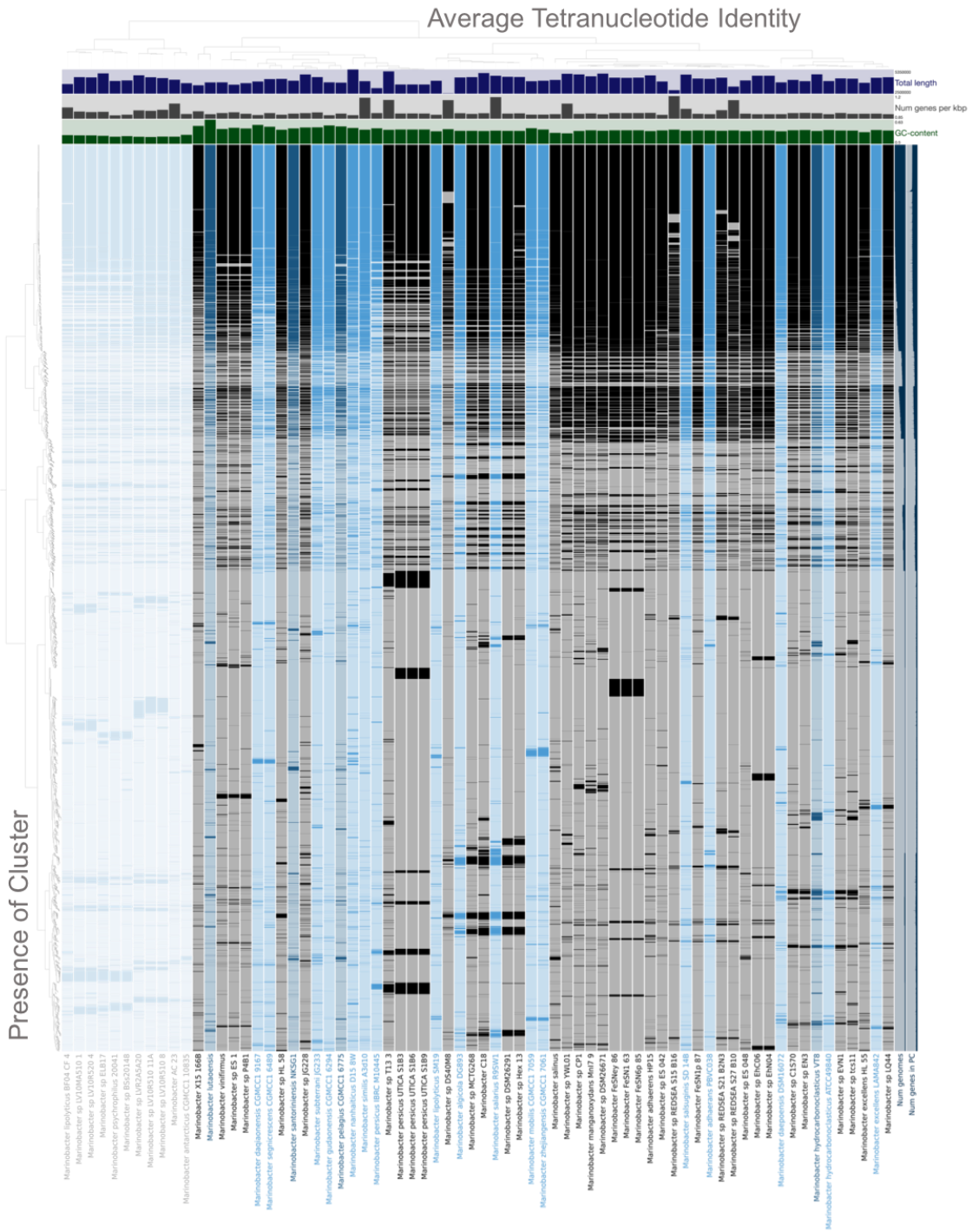


Figure 5.1. Linear graphs of presence and absence of gene clusters in *Marinobacter* strains. Trees were generated using Bray-Curtis dissimilarity. Strains are colored by optimum growth temperature grouping; dark blue, high optimum temperature; blue, moderate optimum temperature; light blue, low optimum temperature; black, no optimum temperature information available.

unanimous and distinct decrease in overall G + C content; averaging 54.20 ±0.42% while the remaining members of the genus average 57.70 ±1.67%, with no overlap between them. It is interesting these cold-adapted strains maintain sufficient AAI percentages to remain within the genus despite the significant difference in G + C content. Previous studies have correlated G + C content with other factors such as nutrient limitation (Mann et al., 2010), however metadata from the isolation of these organisms is insufficient for comparison, and temperature correlation is supported in the literature (Musto et al., 2004). Salinity, pH tolerance, and biome of isolation were also compared using genomic features including G + C content, gene density, genome size, and gene size, though no further correlations were observed.

With the abundance of publically available sequencing data and collaboration of databases, powerful bioinformatics tools allow for the systematic and objective classification of organisms. Public data was utilized to modernize taxonomic classification of the *Marinobacter* from limited gene comparison or biochemical analysis to evaluating similarity at the genomic scale. Literature-derived traits and tolerance parameters can guide modern comparative genomics studies for hypothesis development, but care must be taken regarding the differing technical approaches to isolate characterization as these can drastically alter outcomes. As responsible researchers, it is our onus to deposit information with care and curation to maintain the fidelity of shared resources to assure continued utility.

Chapter 6: Conclusions and Future Directions

Chapter 3:

M. subterrani-mediated Fe(II) oxidation proceeds neither as a mechanism by which the cell facilitates work, nor as a product of passive catalysis of cellular components. Fe(II) oxidation is thus facilitated by stoichiometric reaction with the cell or products produced by metabolic processes of the cell. Though it remains indefinitive whether the reaction occurs proximal to the cell, prolonged viability and function of *M. subterrani* during extended incubation under Fe(II)-oxidizing conditions suggests a distal reaction. Proximal reaction would invariably result in entombment of cells by precipitated Fe(III) products, blocking further reaction if not proving entirely lethal. As distal Fe(II) oxidation requires a diffusible reactant from actively metabolizing cells, confounding factors must first be clarified before the mechanism can be elucidated. Despite extensive efforts, the means by which *M. subterrani* is able to grow in defined medium remains unknown. The inability to establish a condition absent of heterotrophic growth limits our ability to interrogate Fe(II) oxidation independently. More importantly, the ability to grow in defined medium without a known added carbon source suggests there remain potentially relevant components in the system we are unable to control or account for. Barring degradation or alternative reactions, it seems unlikely Fe(II) oxidation is mediated by soluble reactive sulfur or nitrogen species. Though assessment did not include reactions of Fe(II) with gaseous nitrogen species, nitrous oxide generation in heterotrophic culture was not observed, and thus is unexpected to be produced under Fe(II) oxidizing conditions. The biochemical mechanism resulting in the production of

extracellular superoxide remains enigmatic as well, and warrants further study independent of, and in addition to, potential superoxide facilitated Fe(II)-oxidation.

Deletion of genes potentially involved in production of reactive sulfur, nitrogen, or oxygen species failed to reduce the Fe(II) oxidative phenotype of *M. subterrani*, though this fails to eliminate them as candidate mechanisms of action. Additionally, the deletion of genes containing motifs generally ascribed to electron transfer capabilities and in a favorable genetic context also failed to effect Fe(II) oxidation. Fe(II) oxidation in *M. subterrani* may be the cumulative effect of multiple contributing factors, and we lack reliable and quantitative comparison of Fe(II) oxidation rates between strains. Assaying deletion mutants in combination may yield better results, though it is likely the gene encoding the primary contributing factor remains unknown. A member of the genus *Marinobacter* incapable of Fe(II) oxidation is yet to be isolated, and given their prevalence and ubiquitous nature, the geobiochemical implications of determining the exact mechanism of Fe(II) oxidation remain relevant.

Chapter 4:

A single Tn-seq experiment yields a tremendous quantity of information, though when not replicated can only appropriately be utilized for hypothesis generation. Even so, the validity of the library is supported by the genes lacking any mapped reads, and thus can be assumed essential for growth under the rich medium used in library construction. Utility can also be ascertained by similar impact scores associated with gene products sharing quaternary structure or function as a complex. Protein complexed scoring as essential under aerobic growth in rich medium include ATP synthase, cell division

proteins, DNA polymerase III, and ribosomal proteins. Additionally, entire complexes were found to confer benefits when disrupted, including several transporters and type IV fimbriae biogenesis. Confirmation of impacts scores by more empirical means such as gene deletion and complementation and competitive growth assays is certainly required to draw significant conclusions, though these results are sufficient to establish Tn-seq as a functional tool in *M. subterrani*. This approach could certainly prove beneficial to determining the yet undermined factor in defined media allowing growth of *M. subterrani* without additional carbon source, and is recommended before further work is done to this end.

The attempt to develop DEATH-seq as an approach to determine important environmental factors experienced by *M. subterrani* in native conditions may not have resolved into clear hypotheses, but certainly clarified several difficulties which must be addressed before use. While the risk of contaminating the native environment, or introducing genetically modified organisms are paramount, replication of the immensely complex and fluctuating parameters microbes experience in the Soudan Iron Mine cannot be reliably reproduced *ex situ*. Future work with DEATH-seq as an environmental tool are therefore limited to environments described in entirety or *in situ* applications. Additional factors contributing to unintentional mutation, biases in recovery of viable cells, and elimination of DNA from non-viable cells must also be considered for accurate representation. DEATH-seq remains a potentially valuable tool for the analysis of traits contributing to survival, and development should certainly be continued.

Adapting and applying the genetic techniques optimized in *M. subterrani* to additional members of the genus yielded verified transposon mutant libraries. While

additional sequencing depth may be required to provide more substantiated comparison of essential gene profiles between species, cursory manual comparison of computationally annotated results suggests the primary reason for underestimation of the essential core genome to be computational rather than experimental. While GET-HOMOLOGUES provides a powerful tool in proteomic comparison, a clustering algorithm better suited to analysis of genomes derived using varied sequencing, construction, and annotation platforms would provide more accurate results. Manual comparison of output orthologue clusters to determine the degree of erroneously binned genes is required before analysis can be conducted, or the validity of the technique can be verified.

Chapter 5:

Genomes of 71 *Marinobacter* strains were collect from public and private collections and compared using average nucleotide identity (ANI) and average amino acid identity (AAI) to determine phylogenetic relatedness (Rodriguez and Konstantinidis, 2014). As compared to the literature and database derived taxonomy, 21 strains lacking taxonomic speciation were identified to a previously existing species classification. An additional 7 species were identified as having been assigned a species grouping erroneously, and more appropriate species assignments have been suggested based on currently accepted standards. Comparative genomic analysis based on literature-derived tolerance parameters of temperature, salinity, pH, and biome of isolation was conducted using the GET-HOMOLOGUES and Anvi'o software packages. Results suggest while genes may be identified as exclusive and distinct to a grouping, the extreme tolerance

range of *Marinobacter* as a genus is derived from cumulative and stochastic acquisition of contributing factors, rather than defined gene clusters conferring tolerance phenotype. Genome properties including genome size, G + C content, gene density, and average gene length were also compared by tolerance groupings. The clade associated with lower temperature growth optima root deeply and distinctly from the rest of the genus, and contain significantly lower G + C content; though it is unclear whether this confers evolutionary advantage at low temperatures, or these strains share a similarly low G + C content ancestor.

This work demonstrates the utility of publically available sequence information and bioinformatic tools. As additional sequences are deposited to public repositories, automated taxonomic identification based on current standards is an achievable method to assure a measure of quality database maintenance. Regrettably, much publically available data are of limited use due to poor metadata entry and grooming prior to submission. However, until there is incentive to devote labor to assuring quality data are submitted this is unlikely to improve. As new sequencing data advances representation across phylogenies towards confluence, continued work towards reassessment of relatedness will grant better insights as to how environment effects evolution and contributing genetic traits.

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